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(54) Title: METHOD OF INHIBITING LEUKOCYTE ADHESION TO FIBRINOGEN

(57) Abstract

A method is provided for reducing or preventing the adhesion of polymorphonuclear (PMN) leukocytes to fibrinogen such as might occur at the situs of a vascular injury due to disease or a surgical operation such as balloon angioplasty or a vascular transplantation by administration of a composition containing an effective amount of a Clf40 or Clf41 protein from the ligand-binding region of the ClfA protein from staphylococcal bacteria. The use of Clf40 or Clf41 compositions can inhibit adhesion to fibrinogen at the endothelial level so as to treat or prevent undesirable conditions associated with vascular injury, including the development of atherosclerotic plaque or inflammation. In addition, the present invention provides methods of isolating and purifying the Clf40 and Clf41 regions, as well as methods of using compositions containing these proteins in the treatment or prevention of infectious irritations or disease conditions caused by staphylococcal bacteria. Further, the invention contemplates the development and use of vaccines and antibodies based on the Clf40 and Clf41 protein regions, and the use of Clf40 and Clf41 in various methods including the generation of an immune response against these proteins, the enhancement of the immune response against staphylococcal bacteria, and the increase in the phagocytic capacity in the host to counter infection.

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METHOD OF INHIBITING LEUKOCYTE ADHESION TO FIBRINOGEN

BACKGROUND OF THE INVENTION

(1) FIELD OF THE INVENTION

The present invention relates in general to a method of inhibiting leukocyte adhesion to fibrinogen, and in particular to the isolation and use of ligand-binding regions Clf40 and Clf41 from staphylococcal bacteria in methods of inhibiting or preventing the adhesion of polymorphonuclear leukocytes to endothelial tissue so as to inhibit the build up of neutrophils and prevent or reduce inflammation following vascular injury or vascular surgery. In addition, the present invention provides compositions based on the Clf40 or Clf41 regions and their use in generating or enhancing an immune response against staphylococcal bacteria.

(2) DESCRIPTION OF THE RELATED ART

Staphylococcal bacteria, such as *Staphylococcus aureus*, are important human pathogens that can cause diseases both in healthy individuals and in hospital patients. These range from superficial skin infections to life-threatening diseases including endocarditis, osteomyelitis and septic arthritis. Unfortunately, over the years, many traditional methods used to prevent or control the spread or the severity of disease caused by pathogenic staphylococcal bacterial, primarily antibiotic treatment regimens, are no longer effective. Accordingly, the medical community is constantly looking for other modes by which such infections and other diseases can be controlled.

More recently, it has been learned that the adherence of these bacteria to host matrix components, mediated by bacterial surface adhesins, is the initial critical event in the pathogenesis of most infections. The extra-cellular matrix (ECM) of the host

contains numerous macromolecules, including glycoproteins and proteoglycans, that support cellular adhesion and migration of host cells. These processes involve integrins, a family of heterodimeric cell-surface receptors that recognize specific ECM proteins. It has become increasingly evident that bacteria, including *S. aureus*, also utilize the ECM as substrata for their adhesion by way of a family of adhesins called MSCRAMM (microbial surface components recognizing adhesive matrix molecules)(8) that specifically recognize host matrix components.

One important component of the ECM, also occurring in soluble form in blood plasma, is fibrinogen. *S. aureus* possesses several fibrinogen-binding proteins, one of which (clumping factor, ClfA) is primarily responsible for bacterial adherence to fibrinogen substrata (17). ClfA is the prototype of a recently identified multigene family of putative surface proteins characterized by a common domain composed of a unique serine-aspartate repeat (10)(Fig. 1). The gene for the clumping factor protein ClfA, has recently been cloned, sequenced and analyzed in detail at the molecular level (17, 43). The predicted protein is composed of 933 amino acids. A signal sequence of 39 residues occurs at the N-terminus followed by a 520 residue region (region A), which contains the fibrinogen binding domain. A 308 residue region (region R), composed of 154 repeats of the dipeptide serine-aspartate, follows. The R region sequence is encoded by the 18 basepair repeat GAY TCN GAY TCN GAY AGY in which Y equals pyrimidines and N equals any base. The C-terminus of ClfA has features present in many surface proteins of gram-positive bacteria such as an LPDTG motif, which is responsible for anchoring the protein to the cell wall, a membrane anchor, and positive charged residues at the extreme C-terminus. Additional information concerning fibrinogen and fibrinogen binding proteins is disclosed in co-pending applications, including Ser. Nos. 09/421,868, 09/386,960,

09/386,959, and 09/200,650, and in U.S. Pat. No. 6,008,341, and all of these applications and patent references are incorporated by reference.

Previously, we have shown that ClfA of *S. aureus* shares functional similarities with the platelet integrin, $\alpha_{IIB}\beta_3$. These similarities include the presence of calcium-binding EF-hands that inhibit ligand-binding function and also that both proteins interact with the extreme C-terminus of the fibrinogen γ-chain (18, 24). The notion that ClfA interacts with fibrinogen by a similar mechanism to integrins was strengthened when the ligand-binding region of ClfA was shown to have sequence homology to an integrin-like protein (αInt1p) from *Candida albicans* (9). This protein, which interacts with fibrinogen, also has amino acid sequence similarity with the I-domain of the fibrinogen-binding integrin, αMβ2(Mac-1/CR3), the major integrin of phagocytic cells including polymorphonuclear leukocytes (PMN).

Evidence is now emerging that the interaction of αMβ2 with fibrinogen is important for cell-cell interactions. The αMβ2-fibrinogen interaction has been directly implicated in leukocyte adhesive reactions during immune and inflammatory responses. The engagement of fibrinogen by αMβ2 on activated leukocytes and by ICAM-1 on endothelial cells mediates leukocyte adhesion to the vessel wall and subsequent transmigration into inflamed tissue (13, 14, 28). The binding of fibrinogen/fibrin may also result in adhesion of monocytes and neutrophils at sites of vascular injury, such as atherosclerotic plaques (33). Furthermore, fibrinogen and its derivatives directly promote accumulation of inflammatory cells on bio-material implants in animal models and their depletion may abrogate this response (30, 31).

Recognition of fibrinogen by involves two sites within the γ-chain, γ191-202 (P1) and γ377-395 (P2) (1, 32). Although separated in linear amino acid sequence

γ190-202 and γ377-395 are brought into close proximity by the folding of the γ-chain module (34). Peptide and structural analysis of the P2 binding site suggests that residues 383-395 represents a major recognition site (32). Key residues involved in recognition should be exposed on the surface of the γ-chain module of fibrinogen. Examination of the crystal structure reveals that the side-chains of the residues forming the β-strand, γ380-390, are buried or partially buried. However, amino acid residues positioned beyond this β-strand towards the extreme C-terminus of the γ-chain (γ390-395) are surface exposed and are particularly good candidates to play a critical role in αMβ2-binding activity. In addition, these residues are positioned on the face of the fibrinogen γ-chain module from where the extreme C-terminus, which is a flexible appendage in intact fibrinogen, emerges from the modular structure. Although the flexible nature of the fibrinogen γ-chain C-terminus and its close proximity to the major binding sites have been evidenced, it has not previously been shown that ClfA, by interacting with the γ-chain C-terminus, could come into contact with amino acid residues involved in fibrinogen-interaction or could effect exposure of the P1 and P2 binding sites on the surface of intact fibrinogen via a conformational change. In either case, there exists a distinct need in the field to examine the interaction between ClfA and fibrinogen and utilize this interaction to potentially interfere with fibrinogen-binding activity.

As indicated above, the binding of fibrinogen/fibrin may result in adhesion of leukocytes and neutrophils at sites of vascular injury, and thus may give rise to a detrimental conditions such as the development of atherosclerotic plaques (33). In addition, fibrinogen and its derivatives are believed to directly promote the accumulation of inflammatory cells on surfaces of biomaterial implants in animal

models (30, 31) and at sites of vascular injury, which again may need to be treated and/or alleviated. This has been a particular problem in vascular surgical procedures such as balloon angioplasty wherein vascular injury in the coronary artery leads to neutrophil and platelet activation on plaque following such a procedure. For example, it has been shown (40) that activation of blood cells at the balloon-injured coronary artery plaque occurs, and that neutrophil and platelet activation at the situs of the balloon-injury plaque contributes to abrupt vessel closure and late restenosis after percutaneous transluminal coronary angioplasty.

Moreover, although attempts have been made to counteract the buildup of plaque due to this activation following disease or surgical injury to vascular tissue using antibodies (41, 42), there is still a serious need to develop methods of reducing or preventing the interaction of leukocytes, such as polymorphonuclear leukocytes, with immobilized fibrinogen so as to prevent or alleviate conditions such as plaque development or inflammation at the situs of a vascular injury.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide compositions comprised of purified ligand-binding regions of ClfA, such as Clf40 and Clf41, which can be used in methods of reducing or preventing PMN leukocyte adhesion to fibrinogen which might otherwise lead to undesirable conditions such as the development of vascular plaque or deleterious inflammation at the situs of a vascular injury caused by disease or a surgical operation, such as a balloon angioplasty or other vascular surgery, or following a vascular transplantation or implantation procedure.

It is a further object of the present invention to provide isolated and purified peptide compositions of the Clf40 or Clf41 domains that are useful in methods of inhibiting adhesion of ClfA or PMN leukocytes to fibrinogen present on the surface of host cells or implanted biomaterials.

It is a further object of the present invention to provide a vaccine based on ligand-binding regions of the fibrinogen-binding domain, such as Clf40 or Clf41, which can be used in treating or preventing infection by staphylococcal bacterial such as *Staphylococcus aureus*, and which can be utilized in methods of enhancing the immune response to staphylococcal infection and increasing phagocytosis.

It is still further an object of the present invention to generate antisera and antibodies to the purified ligand-binding regions of ClfA, such as Clf40 and Clf41, which can be useful in methods of inhibiting staphylococcal adhesion to fibrinogen in general and which can inhibit staphylococcal adhesion to fibrinogen interacting with an endothelial monolayer.

These and other objects are provided by virtue of the present invention which comprises methods of isolating the ligand-binding region of ClfA such as Clf40 and Clf41 which can inhibit adhesion to fibrinogen of PMN leukocytes such as may occur at endothelial monolayers so as to treat or prevent undesirable conditions such as the development of vascular plaque or deleterious inflammation at the situs of a vascular injury, such as may result from disease or a surgical operation, e.g., balloon angioplasty. In addition, the present invention provides methods of isolating and purifying the Clf40 and Clf41 regions, as well as methods of using compositions containing these proteins in the treatment or prevention of infectious irritations or disease conditions caused by staphylococcal bacteria, including those associated with fibrinogen binding. Further, the invention contemplates the development and use

of vaccines and antibodies based on the Clf40 and Clf41 protein regions, and the use of Clf40 and Clf41 in various methods including the generation of an immune response against these proteins, the enhancement of the immune response against staphylococcal bacteria, and the increase in the phagocytic capacity in the host to counter infection.

These and other objects of the present invention will be described more fully in, or will become obvious from, the detailed description of the preferred embodiments provided hereinbelow.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

FIG. 1 depicts a schematic model showing the domain organization of *S. aureus* clumping factor (ClfA) wherein S is a signal peptide; A, fibrinogen-binding region; R, repeat region; W, cell-wall spanning region; M, membrane-spanning domain; +, positively-charged tail. The minimum fibrinogen-binding domain is located between residues 221 and 559.

FIG. 2 (and inset FIG. 2A) depicts the inhibition of PMN adherence to immobilized fibrinogen with increasing concentrations of recombinant Clf40-(40-559). Immobilized fibrinogen on glass was pre-incubated with recombinant protein for 1 hr. at 37°C. Unbound protein was removed by washing with PBS. PMN (5×10^5 cells) were incubated with immobilized fibrinogen for 20 min. at 37°C. Adherent PMN were quantified microscopically. The number of PMN binding to fibrinogen in the absence of inhibitors was assigned a value of 100%.

FIG. 2A, the *Inset*, depicts the inhibition of PMN (5×10^5 cells) adhesion to immobilized fibrinogen by recombinant Clf40-(40-559) (3.5μM), Clf41-(221-559).

(5.2μM) and α_M I-domain (3.5μM). Data are expressed as means ± SD (error bars) (*n*=4).

FIG. 3 depicts the inhibition of PMN adherence to glass, immobilized fibronectin and immobilized fibrinogen with recombinant Clf40-(40-559) (3.5μM). Adherent PMN were quantified microscopically. The number of PMN attached to each substrate in the absence of Clf40-(40-559) was assigned a value of 100%. Data are expressed as means ± SD (error bars) (*n* = 4).

FIG. 4 depicts the adherence of PMN to an human umbilical vein endothelial cell monolayer. PMN (5×10^6 cells) were added to immobilized endothelial cells that had been pre-incubated with fibrinogen (1 mg/ml) (A), fibrinogen (1 mg/ml) and Clf40-(40-559) (23.7μM) (B), and Clf40-(40-559) (23.7μM) in the absence of fibrinogen (C). After washing with PBS enriched in Ca^{2+} and Mg^{2+} and fixing in 4% paraformaldehyde in PBS, adherent PMN granules were stained with the benzidine hydrochloride-based Kaplow stain (12). The degree of cell attachment was evaluated microscopically.

FIG. 5 depicts the inhibition of the interaction between biotinylated α_M I-domain and immobilized fibrinogen (5μg/ml) by Clf40-(40-559). Bound protein was detected with alkaline phosphatase-labeled avidin and quantitated colorimetrically. Binding of α_M I-domain in absence of Clf40-(40-559) was assigned a value of 100. Data are expressed as means ± SD (error bars) (*n* = 3).

FIG. 6 depicts the phagocytosis of FITC-*S. aureus* strains by purified human polymorphonuclear leukocytes. *S. aureus* wild-type and mutants were pretreated with 0% NHS (Non-opsonized) or 10% NHS (Opsonized) and then incubated with human PMNs (1×10^6 cells) for 5 minutes at 37°C. The level of phagocytosis of each strain

of *S. aureus* by PMNs was determined by flow cytometry as described in the Method section. Results are expressed as mean ± standard error of the mean (n = 5).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, there is provided a method of preventing adhesion of leukocytes, such as polymorphonuclear (or PMN) leukocytes, to immobilized fibrinogen which comprises administering an effective amount of a composition comprising a Clf40 or Clf41 protein to a human or animal patient in such a manner that it will prevent or reduce the effects, such as development of plaque or inflammation, caused or aggravated by the adhesion of leukocytes to fibrinogen. This may be accomplished in a variety of ways including administration of one or more of these proteins directly at the situs of the injury or systemically in a manner where the proteins will ultimately work at the desired location. As would be recognized by one of ordinary skill in this art, the Clf40 or Clf41 proteins as utilized in the present invention would encompass active fragments or portions thereof which effect the inhibition of leukocyte adhesion to fibrinogen in the same manner as the protein in its complete form.

As discussed above, it has been shown that the binding of fibrinogen/fibrin may result in adhesion of leukocytes and neutrophils at sites of vascular injury, and thus may give rise to a detrimental conditions such as the development of atherosclerotic plaques (33). In addition, fibrinogen and its derivatives are believed to directly promote the accumulation of inflammatory cells on surfaces of biomaterial implants in animal models (30, 31) and at sites of vascular injury, which again may need to be treated and/or alleviated. This problem has been particularly observed with regard to vascular surgical procedures such as balloon angioplasty wherein vascular injury in

the coronary artery leads to neutrophil and platelet activation on plaque following such a procedure. In fact, it has been shown (40) that activation of blood cells at balloon-injured coronary artery plaque occurs, and that neutrophil and platelet activation at the situs of the balloon-injury plaque contributes to abrupt vessel closure and late restenosis after percutaneous transluminal coronary angioplasty. Accordingly, the method of the present invention will thus be useful in preventing or alleviating such conditions by achieving the inhibition of adhesion of leukocytes, such as polymorphonuclear (or PMN) leukocytes, to immobilized fibrinogen wherever necessary to treat or prevent these deleterious vascular conditions.

Further, the Clf40 and Clf41 proteins of the present invention may be useful as analogs in the development and/or identification of small molecules which can mimic the ability of Clf40 and Clf41 and thus be utilized in methods for treating or preventing deleterious conditions associated with the adhesion of leukocytes to fibrinogen. In this case, Clf40 and Clf41 proteins may be used to determine more precisely the relevant ClfA binding sites and the relevant peptide sequences involved in such binding so as to allow one to develop smaller molecules which contain these active regions.

In addition, the present invention will be suitable for inhibiting adhesion of leukocytes on biological implants such as those implants utilized in transplants of natural or artificial blood vessels or other organs. Accordingly, the leukocyte adhesion-inhibiting compositions of the present invention will be useful in numerous applications wherein such effects are desired. For example, medical devices or polymeric biomaterials to be treated with the leukocyte adhesion-inhibiting compositions of the present invention may include, but are not limited to, staples, sutures, replacement heart valves, cardiac assist devices, hard and soft contact

lenses, intraocular lens implants (anterior chamber or posterior chamber), other implants such as corneal inlays, kerato-prostheses, vascular stents, epikeratophalia devices, glaucoma shunts, retinal staples, scleral buckles, dental prostheses, thyroplastic devices, laryngoplastic devices, vascular grafts, soft and hard tissue prostheses including, but not limited to, pumps, electrical devices including stimulators and recorders, auditory prostheses, pacemakers, artificial larynx, dental implants, mammary implants, penile implants, cranio/facial tendons, artificial joints, tendons, ligaments, menisci, and disks, artificial bones, artificial organs including artificial pancreas, artificial hearts, artificial limbs, and heart valves; stents, wires, guide wires, intravenous and central venous catheters, laser and balloon angioplasty devices, vascular and heart devices (tubes, catheters, balloons), ventricular assists, blood dialysis components, blood oxygenators, urethral/ureteral/urinary devices (Foley catheters, stents, tubes and balloons), airway catheters (endotracheal and tracheostomy tubes and cuffs), enteral feeding tubes (including nasogastric, intragastric and jejunal tubes), wound drainage tubes, tubes used to drain the body cavities such as the pleural, peritoneal, cranial, and pericardial cavities, blood bags, test tubes, blood collection tubes, vacutainers, syringes, needles, pipettes, pipette tips, and blood tubing.

As will be recognized by one skilled in the art, because of the numerous possible applications of the methods of the present invention, and because the inhibition of leukocyte adhesion by Clf40 is dose-dependent, as described further below, one skilled in the art will recognize that any particular treatment regimen will have to be determined on the basis of the extent and nature of the condition to be treated, and that the amount of the particular protein administered will fall within a range of values which would be suited to treat the particular condition as needed for

the particular application. As indicated above, these conditions primarily include vascular or endothelial injury associated with a variety of pathogenic diseases or a variety of surgical operations ranging from angioplasty and other similar operations to transplantation and the implantation of vascular prosthetic devices.

As would be recognized by one skilled in the art, the Clf40 and Clf41 proteins of the present invention, or active fragments thereof, may be prepared in a number of conventional ways, including isolation and purification of the natural proteins, or more preferably, using recombinant methods well known in the art to produce isolated and purified forms of these proteins. For example, in a particularly preferred embodiment, the ClfA protein compositions of the present invention may be prepared using a bacterial host such as *Escherichia coli* XL-1 Blue for plasmid cloning and protein expression, growing the plasmid-containing bacterial host in suitable nutrient media containing desired additives such as Ampicillin. Next, expression plasmids may be cloned in a suitable manner known in the art, which in the preferred embodiment comprises using amplified fragments of the *ClfA* gene cloned into an expression plasmid such as pQE30 (Qiagen Inc.) to generate the constructs. In accordance with the present invention, the ligand-binding regions Clf40 and Clf41 are preferably prepared using plasmids pCF40-(40-559) and pCF41-(221-559), respectively, as has been described previously in references (see, e.g., Ref no. 24) which are incorporated herein. The recombinant protein expressed from these vectors contains an N-terminal extension of six histidine residues.

The recombinant proteins of the present invention can then be expressed and purified once again using conventional techniques well known in the art, such as by expressing of the plasmids in a bacterial host followed by purification in an appropriate chromatographic apparatus. In the preferred embodiment, the

recombinant Clf40-(40-559) of the invention can be expressed in a bacterial host such as *E. coli* XL-1 Blue and purified by immobilized metal chelate affinity chromatography and ion-exchange chromatography as described previously (24). Analysis by sodium dodecyl sulfate-polyacrylamide electrophoresis with Coomassie blue staining and Western immunoblotting indicated that a single immunoreactive protein of the correct molecular weight was obtained. In addition, the recombinant Clf41-(221-559) can be expressed and purified in the same way. For testing purposes, the glutathione S-transferase-α_M I-domain fusion protein was purified by glutathione-Sepharose (Pharmacia Biotech Inc.) affinity chromatography and cleaved with bovine thrombin according to manufacturers instructions.

In the preferred embodiment, the Clf40 or Clf41 proteins of the present invention, or active portions or fragments thereof, as well as antibodies as will be discussed further below, may be formulated in combination with a suitable pharmaceutical vehicle, excipient or carrier such as would be well known in this art. Examples of such suitable pharmaceutical vehicles, excipients or carriers include saline, dextrose, water, glycerol, ethanol, other therapeutic compounds, and combinations thereof. The formulation should be appropriate for the mode of administration, and should be compatible with the preferable use of the compositions of the invention, namely methods of treatment of conditions associated with vascular disease or surgical injury such as inflammation and the development of atherosclerotic plaque.

In addition to the use of the isolated, recombinant or synthetic proteins of the present invention, or antigenic portions thereof (including epitope-bearing fragments), or fusion proteins including the Clf40 or Clf41 proteins in the therapeutic leukocyte adhesion-inhibiting manner as described above, the proteins, or antigenic portions

thereof, can be utilized to generate antibodies, which can then be isolated, purified and/or further utilized or administered for therapeutic purposes or for detection and determination of staphylococcal infections. Accordingly, in accordance with the invention, there is provided a method of generating an immune response to the Clf40 and Clf41 proteins using conventional means well known in this field to generate such a response. For example, an immune response may be produced when the immunogen (e.g., Clf40, Clf41 or an immunogenic fragment or portion thereof) is injected into humans or animals, including mice, rabbits, rats, goats, sheep, guinea pigs, chickens, and other animals.

Further, to enhance immunogenicity, the proteins may be conjugated to a carrier molecule. Suitable immunogenic carriers include proteins, polypeptides or peptides such as albumin, hemocyanin, thyroglobulin and derivatives thereof, particularly bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), polysaccharides, carbohydrates, polymers, and solid phases. Other protein derived or non-protein derived substances are known to those skilled in the art. An immunogenic carrier typically has a molecular weight of at least 1,000 Daltons, preferably greater than 10,000 Daltons. Carrier molecules often contain a reactive group to facilitate covalent conjugation to the hapten. The carboxylic acid group or amine group of amino acids or the sugar groups of glycoproteins are often used in this manner. Carriers lacking such groups can often be reacted with an appropriate chemical to produce them. Alternatively, a multiple antigenic peptide comprising multiple copies of the protein or polypeptide, or an antigenically or immunologically equivalent polypeptide may be sufficiently antigenic to improve immunogenicity without the use of a carrier.

The Clf40 or Clf41 proteins (which encompasses or immunogenic fragments or portions thereof) or appropriate combination of proteins, may also be administered with an adjuvant in an amount effective to enhance the immunogenic response against the conjugate. For example, one such adjuvant widely used in humans has been alum (aluminum phosphate or aluminum hydroxide). Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants used in research and veterinary applications may also be employed, but these adjuvants have toxicities which limit their potential use in human vaccines. However, suitable chemically defined preparations include muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates or encapsulation of the conjugate within a proteoliposome (such as those described in References 44 and 45, below, and incorporated herein by reference), and encapsulation of the protein in lipid vesicles such as Novasome™ lipid vesicles (Micro Vescular Systems, Inc., Nashua, NH) may also be useful.

In addition, the present invention may be utilized as immunological compositions, including vaccines, and other pharmaceutical compositions containing the Clf40 or Clf41 proteins or portions thereof are included within the scope of the present invention. Either one or both of the Clf40 or Clf41 proteins, or active or antigenic fragments thereof, or fusion proteins thereof, can be formulated and packaged, alone or in combination with other antigens, using methods and materials known to those skilled in the art for vaccines. The immunological response may be used therapeutically or prophylactically and may provide antibody immunity or cellular immunity, such as that produced by T lymphocytes.

The term "vaccine" as used herein includes vaccines prepared from the Clf40 or Clf41 compositions of the present invention as well as DNA vaccines in which the nucleic acid molecule encoding the ligand-binding regions of the present invention is

used in a pharmaceutical composition is administered to a patient. For genetic immunization, suitable delivery methods known to those skilled in the art include direct injection of plasmid DNA into muscles, delivery of DNA complexed with specific protein carriers, coprecipitation of DNA with calcium phosphate, encapsulation of DNA in liposomes, particle bombardment, and *in vivo* infection using cloned retroviral vectors.

In a preferred embodiment, a vaccine is packaged in a single dosage for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or nasopharyngeal (i.e., intranasal) administration. The vaccine is most preferably injected intramuscularly into the deltoid muscle. The vaccine is preferably combined with a pharmaceutically acceptable carrier to facilitate administration. The carrier is usually water or a buffered saline, with or without a preservative. The vaccine may be lyophilized for resuspension at the time of administration or in solution. The vaccine may additionally contain stabilizers or pharmaceutically acceptable preservatives, such as thimerosal (ethyl(2-mercaptobenzoate-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO).

The immunological compositions, such as vaccines, and other pharmaceutical compositions can be used alone or in combination with other blocking agents as appropriate to protect against human and animal infections caused by or exacerbated by staphylococci. The compositions thus may protect humans or ruminants against various staphylococcal infections, and the vaccine may also be useful in protecting other species of animals, for example canine and equine animals, against similar staphylococcal infections.

In accordance with the invention, the antibodies to Clf40 or Clf41, or to fragments thereof, such as described above, can also be used for the specific detection of fibrinogen-binding staphylococcal proteins, for the prevention of infection from staphylococci, for the treatment of an ongoing infection, or for use as research tools. The term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, including the products of an Fab immunoglobulin expression library. Generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art. In the present case, specific polyclonal antiserum utilizing the proteins of the present invention may be generated and used for specific agglutination assays to detect bacteria which express the proteins of the present invention.

Additionally, any of the above described antibodies may be labeled directly with a detectable label for identification and quantification of staphylococci. Labels for use in immunoassays are generally known to those skilled in the art and include enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances, including colored particles such as colloidal gold or latex beads. Suitable immunoassays include enzyme-linked immunosorbent assays (ELISA).

Alternatively, the antibody may be labeled indirectly by reaction with labeled substances that have an affinity for immunoglobulin. The antibody may be conjugated with a second substance and detected with a labeled third substance having an affinity for the second substance conjugated to the antibody. For example, the antibody may be conjugated to biotin and the antibody-biotin conjugate detected using labeled avidin or streptavidin. Similarly, the antibody may be conjugated to a hapten and the antibody-hapten conjugate detected using labeled anti-hapten

antibody. These and other methods of labeling antibodies and assay conjugates are well known to those skilled in the art. Antibodies to the fibrinogen-binding proteins Clf40 or Clf41, or portions thereof, may also be used in production facilities or laboratories to isolate additional quantities of the proteins, such as by affinity chromatography.

In addition to the methods described above, it is also the case that the interaction of leukocytes such as PMN with immobilized fibrinogen plays an important role in the host immune response against pathogenic bacteria, and thus the present invention can be utilized in methods of enhancing the immune response against such infectious bacteria. As indicated above, this interaction is important in the inflammatory response because of the contribution of leukocyte adhesion to the endothelium and subsequent transmigration to sites of infection (see, e.g., references 13, 14, and 28 below). In order for PMN to initiate a respiratory burst in response to soluble cytokines, they are required to utilize their β2 integrins (including αMβ2) and interact with immobilized matrix molecules such as fibrinogen (22). Furthermore, occupancy of αMβ2 with immobilized fibrinogen modulates monocyte oxidative response and phagocytosis of opsonized particles (31). Accordingly, the Clf40 and Clf41 protein compositions of the present invention may be utilized as appropriate by causing an enhancement in the immune response against staphylococcal bacteria, and in addition may promote opsonization and phagocytosis of staphylococcal bacteria in the host. In such methods, an amount of an appropriate composition in accordance with the invention, such as an antibody composition comprising antibodies generated to Clf40 or Clf41 in an amount effective to promote opsonization and phagocytosis of staphylococcal bacteria, or a Clf40 or Clf41 protein composition sufficient to generate an immune response thereto so as to enhance the

immunogenic response against staphylococcal bacteria, is effectively administered to the patient.

Further, the Clf40 and Clf41 proteins and compositions in accordance with the present invention can be used to screen antibodies or antisera for hyperimmune patients from whom can be derived specific antibodies having a very high affinity for the proteins. Further, the present invention contemplates use in the detection of the presence of Clf40 or Clf41 or their antibodies and the diagnosis of related staphylococcal diseases ranging from superficial skin infections to life-threatening diseases including endocarditis, osteomyelitis and septic arthritis. In accordance with the invention, a preferred method of detecting the presence of Clf40 or Clf41 proteins involves the steps of obtaining a sample suspected of containing staphylococci. The sample may be taken from an individual, for example, from one's blood, saliva, tissues, bone, muscle, cartilage, or skin, and the sample may be screened for the presence of Clf40, Clf41 or associated proteins or fragments using the compositions described above via suitable methods well known in the art.

The invention further contemplates a kit containing one or more Clf40 or Clf41-specific nucleic acid probes, which can be used for the detection of fibrinogen-binding proteins from staphylococci in a sample, or for the diagnosis of staphylococcal bacterial infections. A kit for the detection and/or diagnosis of staphylococcal infection is thus provided in accordance with the invention which comprises a means to identify the presence of Clf40 or Clf41 proteins in the sample, e.g., an antibody for Clf40 or Clf41 as described above, and appropriate means such as a label by which the sample can be detected is binding of the antibody occurs. Such a kit can also contain the appropriate reagents for hybridizing the probe to the sample and detecting bound probe. In an alternative embodiment, the kit contains antibodies specific to either or

both Clf40 and Clf41 proteins or active portions thereof which can be used for the detection of staphylococci.

In yet another embodiment, the kit contains either or both the Clf40 and Clf41 proteins, or active fragments thereof, which can be used for the detection of staphylococcal bacteria or for the presence of antibodies to fibrinogen-binding staphylococcal proteins in a sample. The kits described herein may additionally contain equipment for safely obtaining the sample, a vessel for containing the reagents, a timing means, a buffer for diluting the sample, and a colorimeter, reflectometer, or standard against which a color change may be measured.

In a preferred embodiment, the reagents, including the protein or antibody, are lyophilized, most preferably in a single vessel. Addition of aqueous sample to the vessel results in solubilization of the lyophilized reagents, causing them to react. Most preferably, the reagents are sequentially lyophilized in a single container, in accordance with methods well known to those skilled in the art that minimize reaction by the reagents prior to addition of the sample.

The isolated proteins of the present invention, or active fragments thereof, and antibodies to the proteins may be useful for the treatment and diagnosis of staphylococcal bacterial infections as described above, or for the development of anti-staphylococcal vaccines for active or passive immunization. Further, when administered as pharmaceutical composition to a situs where a vascular injury has occurred, whether from pathogenic disease, surgical operation or transplantation, the protein compositions of the present invention will be useful in inhibiting adhesion of leukocytes to the fibrinogen at that site which may be helpful in avoiding the development of atherosclerotic plaques or reducing deleterious inflammation.

When labeled with a detectable biomolecule or chemical, the fibrinogen-binding proteins described herein are useful for purposes such as *in vivo* and *in vitro* diagnosis of staphylococcal infections or detection of staphylococcal bacteria. Laboratory research may also be facilitated through use of such protein-label conjugates. Various types of labels and methods of conjugating the labels to the proteins are well known to those skilled in the art. Several specific labels are set forth below. The labels are particularly useful when conjugated to a protein such as an antibody or receptor. For example, the protein can be conjugated to a radiolabel such as, but not restricted to, ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or ^{131}I. Detection of a label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the protein by conventional methods, and the labeled protein is detected when an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light. Fluorogens may also be used to label proteins. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The protein can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the protein can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. For example, the protein can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as Luminol®) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol)

(Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogeneic or fluorogenic dioxetane derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, proteins may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art. The location of a ligand in cells can be determined by labeling an antibody as described above and detecting the label in accordance with methods well known to those skilled in the art, such as immunofluorescence microscopy.

The following examples are provided which relate to various aspects of the development and use of the preferred embodiments of the present invention, and it should be appreciated by those of skill in the art that the techniques disclosed above and in the examples below are merely exemplary of the compositions and methods of the present invention. Accordingly, one of ordinary skill in this art will recognize that numerous alterations and amendments may be made to the embodiments disclosed in the present application which will still be considered to fall within the scope of the present invention.

EXAMPLES

EXAMPLE 1: PREPARATION OF CLFA PROTEIN COMPOSITIONS AND ASSAYS TO DETERMINE THEIR EFFECT ON PMN ADHESION TO FIBRINOGEN

Bacterial strains and growth conditions.

Escherichia coli XL-1 Blue (3) was used as the bacterial host for plasmid cloning and protein expression. *E. coli* cells harboring plasmids were routinely grown in L-broth, Terrific broth, and L-agar (27). Ampicillin (100 μg/ml) was incorporated as appropriate.

Construction of expression plasmids.

Amplified fragments of the *ClfA* gene were cloned into the expression plasmid pQE30 (Qiagen Inc.) to generate the constructs pCF40-(40-559) and pCF41-(221-559) as described previously (24). Recombinant protein expressed from this vector contains an N-terminal extension of six histidine residues.

Expression and purification of recombinant proteins.

Recombinant Clf40-(40-559) was expressed in *E. coli* XL-1 Blue and purified by immobilized metal chelate affinity chromatography and ion-exchange chromatography as described previously (24). Analysis by sodium dodecyl sulfate-polyacrylamide electrophoresis with Coomassie blue staining and Western immunoblotting indicated that a single immunoreactive protein of the correct molecular weight was obtained (data not shown). Recombinant Clf41-(221-559) was expressed and purified in the same way. The glutathione S-transferase-α_M I-domain fusion protein was purified by glutathione-Sepharose (Pharmacia Biotech Inc.) affinity chromatography and cleaved with bovine thrombin according to manufacturers instructions.

PMN adherence assay.

Measurement of the adhesion of PMN to immobilized fibrinogen was carried out as described previously with minor modification (16). 100μl volumes were used for all additions. Briefly, glass slides were coated with purified fibrinogen (200μg/ml) for 1 h at 37°C in a moist chamber. Unbound fibrinogen was removed by washing in PBS and slides were post-coated with 1% human serum albumin for 2 h at 37°C. Purified

human PMN isolated from whole blood from healthy donors (5 x 10^5 cells/ml in medium 199) (2) were allowed to adhere to immobilized fibrinogen for 20 min at 37°C. This incubation period was selected on the basis of time course studies (data not shown). The slides were then dip-washed five times in PBS to remove non-associated PMN cells. Following rinsing, specifically attached cells were quantitated microscopically (with photography) by direct analysis of selected fields. For inhibition experiments, the fibrinogen-coated slides were pre-incubated with various concentrations of Clf40-(40-559), Clf41-(221-559) and α_M I-domain for 2 h at 37°C. Unbound protein was removed by three washes with PBS followed by addition of PMN (5 x 10^5 cells). In variations of the above assay, the inhibitory potential of Clf40-(40-559) on adherence of PMN to glass alone and to glass slides coated with fibronectin (200µg/ml) were also investigated. Coating conditions were performed as for fibrinogen-coated glass slides.

Endothelial cell-PMN adhesion assay.

Human endothelial cells (HUVEC) were obtained from umbilical cord veins using 0.1% collagenase in Krebs-Ringer bicarbonate buffer. The cells were plated in 60 mm gelatin-coated (0.1% for 2 hours) Petri dishes and grown in RPMI 1640 (Gibco) supplemented with 50 units/ml penicillin, 50 g/ml streptomycin, 10% fetal calf serum and 15 µg/ml endothelial cell growth supplement. Cells were trypsinized briefly and plated in gelatinized-24 wells culture plate as described above. Generally, second-passage cells in monolayer culture were used for experiments, at confluence. After rinsing twice in PBS supplemented by Ca^{2+} and Mg^{2+}, cells were incubated with 1mg/ml human fibrinogen for 1 h at 37°C. Unbound fibrinogen was removed by

washing in PBS supplemented by Ca^{2+} and Mg^{2+}. For inhibition experiments, recombinant Clf40-(40-559) (23.7μM) was incubated with the fibrinogen-coated endothelial cell monolayer for 30 min at 37°C. As a control, Clf40-(40-559) was also incubated with the endothelial cells in the absence of fibrinogen. Human PMNs (5 X 10^6 cells) were then added to each well. After an incubation period of 30 min at 37°C, each well was rinsed several times with PBS enriched in Ca^{2+} and Mg^{2+} to remove non-associated PMN, and fixed in 4% paraformaldehyde in PBS. The cells were washed with 0.9% NaCl and stained for 90 sec with the benzidine hydrochloride-based Kaplow stain (12). The staining solution was washed away prior to photography. Adherent neutrophil granules were stained for their contents of MPO with the reagent of Kaplow and appeared dark brown/black under visible light.

αM I-domain-fibrinogen binding assay.

To confirm the ability of recombinant Clf40-(40-559) to interfere with fibrinogen-binding function, a cell-free αM I-domain-fibrinogen binding assay was developed. Fibrinogen (5μg/ml) in coating solution (0.02% sodium carbonate buffer (pH 9.6)) was absorbed onto 96-well enzyme-linked immunosorbent assay microtiter plates (Sarstedt) for 16 h at 4°C. The plates were washed three times with PBS with 0.05% Tween 20 (PBS-T). A solution of 5% bovine serum albumin (BSA) was added to the wells to block any remaining protein-binding sites. After 2 h at 37°C, the well were washed again three times with PBS-T. Recombinant αM I-domain, which had previously been biotinylated (with sulfo-NHS-biotin (Pierce)), was added and incubated for 90 min at room temperature. Wells were washed, incubated with alkaline phosphatase-coupled avidin, washed again, developed with substrate, and

quantified colorimetrically using a microplate reader. For the inhibition experiments, the immobilized fibrinogen was pre-incubated with increasing concentrations of Clf40-(40-559) for 1 h at room temperature. Unbound protein was removed by three washes with PBS-T.

EXAMPLE 2: ANALYSIS OF THE EXPERIMENTS OF EXAMPLE 1 AND FURTHER TESTING REGARDING THE EFFECT OF CLF40 AND CLF41 ON PMN ADHESION TO FIBRINOGEN

ClfA inhibits PMN leukocyte adhesion to fibrinogen.

Recombinant forms of ClfA encompassing full length region A (Clf40, residues 40-559) or the smallest truncated protein that maintained fibrinogen-binding activity (Clf41, residues 221-559) were tested for their ability to inhibit the adhesion of human PMN to immobilized fibrinogen. The recombinant ClfA proteins were pre-incubated with immobilized fibrinogen. Unbound protein was removed by washing and the PMN were allowed to interact. As shown in Fig. 2, Clf40-(40-559) inhibited PMN adhesion to fibrinogen in a dose-dependent manner. At the highest concentrations of Clf40-(40-559) tested, PMN adherence was reduced to 20%. of that observed in the absence of inhibitor (n = 4). Interestingly, the minimum fibrinogen-binding truncate of ClfA, Clf41-(221-559), was much less effective at inhibiting PMN adherence to fibrinogen (Fig. 2, inset) even at high concentrations. Several independent studies and approaches have implicated the I-domain of the α_M subunit in the recognition of fibrinogen by $\alpha_M\beta_2$, the major integrin of leukocytes (5, 21, 35). To confirm that activated PMN adherence to fibrinogen is mediated primarily via the integrin, the recombinant I-domain of the α_M subunit was purified and tested for its ability to inhibit PMN fibrinogen-binding activity. As expected, increasing concentrations of recombinant I-domain caused a dose-dependent inhibition of the adhesion of PMN to immobilized fibrinogen (data not

shown). At 3.5μM, adhesion to fibrinogen was reduced by 80% (Fig. 2, inset) ($n = 4$).

To demonstrate that Clf40-(40-559) was interfering with fibrinogen-mediated attachment, the ability of the protein to interfere with attachment of activated PMN to fibronectin and glass was analyzed. Consistent with the specificity of inhibition of fibrinogen-mediated adherence, Clf40-(40-559) had no effect on leukocyte adherence to fibronectin-coated slides or to glass alone (Fig. 3). Finally, none of the inhibitors analyzed affected cell viability as assessed by trypan blue exclusion.

ClfA inhibits PMN leukocyte adhesion to fibrinogen.

Fibrinogen bound to ICAM-1 on endothelial cells can facilitate the bridging of these cells to on leukocytes (6, 13). This interaction has been implicated in the inflammatory response by contributing to leukocyte adhesion to the endothelium and subsequent transmigration (14). In addition, Cheung et al. (1991) (4) demonstrated that fibrinogen can act as a bridging molecule in the adherence of *S. aureus* to endothelial cells and implicated the fibrinogen-binding receptor of *S. aureus* as an important factor in this process. We addressed the question of whether ClfA, the major fibrinogen-binding protein of *S. aureus*, could inhibit the attachment of PMN to fibrinogen bound to cultured human umbilical vein endothelium. PMN were allowed to adhere to an endothelial cell monolayer that had been pre-incubated with fibrinogen. The effect of Clf40-(40-559) on this adherence was evaluated microscopically. As shown in Fig. 4A, endothelial cells with bound fibrinogen supported efficient leukocyte adhesion. Inclusion of Clf40-(40-559) protein resulted in a dramatic reduction in leukocyte attachment to (Fig. 4B). In the absence of fibrinogen, there was reduced PMN adhesion to the endothelium probably mediated by ICAM-1 which can interact

directly with αMβ2 and αLβ2 on leukocytes (Fig. 4C). Clf40-(40-559) had no effect on this fibrinogen-independent endothelial-leukocyte interaction.

ClfA inhibits the interaction between the I-domain of the αM subunit and fibrinogen.

Many studies have shown that the I-domain of the α-subunit of αMβ2 is an independent structural and functional unit and has many of the binding functions of the intact receptor (15). To further establish the molecular basis of ClfA inhibition of PMN adhesion to fibrinogen, we investigated the inhibitory activity of Clf40-(40-559) on the interaction between recombinant αM I-domain and immobilized fibrinogen. As shown in Fig. 5, Clf40-(40-559) inhibited the binding of biotinylated αM I-domain to fibrinogen in a dose-dependent manner. At 5μM concentration, Clf40-(40-559) reduced binding by 60%. In contrast, the control protein (BSA) had no effect on αM I-domain-fibrinogen interaction. This result indicates that the inhibitory effect of ClfA on PMN leukocyte adherence to fibrinogen is mediated primarily via its effect on the fibrinogen-binding activity of αMα2.

In light of these studies, it has now been shown that the fibrinogen-binding protein of *S. aureus*, ClfA, is an effective and specific inhibitor of PMN interaction with immobilized fibrinogen. Furthermore, the data presented here indicates that ClfA can interfere with PMN fibrinogen-binding activity by interacting with the fibrinogen γ-chain at, or in close proximity, to the binding sites. The three-dimensional structure of the fibrinogen γ-chain module, which became available recently (34), revealed that the two amino acid sequences implicated in interaction (P1, residues 190-202, and P2,

residues 377-395) reside adjacent to one another as part of two anti-parallel β-strands. Thus, although the P1 and P2 sites are separated by 178 residues in terms of linear amino acid sequence, the specific folding of the γ-chain module brings these two recognition sequences into close proximity to form a complex ligand-binding site. The known binding site in fibrinogen for ClfA occurs at the C-terminus of the γ-chain (18). This segment of the structure emerges as an appendage from the globular portion of the γ-chain module at the P1 and P2 sites and is in close proximity to the P2 site. In addition, structural NMR and crystallographic studies provide firm evidence that the C-terminal region of the γ-chain is conformationally flexible which may be functionally important in its biological context (34). It thus appears that when ClfA interacts with the C-terminus of the γ-chain, the protein interferes with access to the P1 and P2 binding sites. This interference may be via steric hindrance blocking accessibility to the recognition sequences.

Alternatively, ClfA may interact directly with one or either of these sites and prevent interaction due to molecular mimicry between the two fibrinogen-binding proteins. Previously, we have shown that ClfA is a potent inhibitor of $\alpha_{IIB}\beta_3$-mediated interaction of platelets with fibrinogen via a molecular mimicry mechanism (18). Our preliminary data indicates that ClfA can recognize a sequence in the fibrinogen γ-chain other than the extreme C-terminus (25). Furthermore, these data suggest that only the recombinant truncate encompassing the full ligand-binding region, Clf40-(40-559), has the capacity to interact with the additional site(s). The minimum fibrinogen-binding truncate, Clf41-(221-559), binds exclusively to the γ-chain C-terminus. Consistent with this proposal is the observation that Clf41-(221-559), while still able to inhibit interaction between PMN and fibrinogen (Fig. 2, inset), had a far reduced ability

when compared to ability of Clf40 to inhibit the interaction between PMN and fibrinogen.

The importance of the interaction between PMN and immobilized fibrinogen in host immune response to infection is well established. Firstly, the interaction is considered crucial in the inflammatory response by contributing to leukocyte adhesion to the endothelium and subsequent transmigration to sites of infection (13, 14, 28). Secondly, in order for PMN to initiate a respiratory burst in response to soluble cytokines, they are required to utilize their β_2 integrins (including $\alpha M\beta 2$) and interact with immobilized matrix molecule such as fibrinogen (22). Furthermore, occupancy of with immobilized fibrinogen profoundly modulates monocyte oxidative responses and phagocytosis of opsonized particles (31). Lastly, fibrinogen and its derivatives are known to directly promote the accumulation of inflammatory cells on biomaterial implants (29, 30). Interestingly, it is well established that biomaterial implants are important sites for the initiation of Staphylococci infection (7). In light of the crucial role PMN play in host response to infection by *S. aureus* and, in particular, the importance of leukocyte interaction with fibrinogen, it is reasonable to suggest that the ability of bacteria to interfere with this interaction would be advantageous and could influence the pathogenic potential of infecting microorganisms. A role for ClfA in pathogenesis has been demonstrated in a rat endocarditis model (20). A *ClfA* mutant of *S. aureus* Newman had a significantly reduced ability to cause infection compared to the wild-type strain. Similarly, a ClfA mutant tested in a mouse septic arthritis model demonstrated a reduced ability to cause arthritic disease (11). Undoubtedly, reduction in fibrinogen-binding activity plays a major role in the compromised pathogenicity of the *S. aureus ClfA* mutant strains. However, it is possible that reduced ability to

interfere with fibrinogen-mediated PMN function in host response to infection may contribute to the decreased virulence of the mutant strains.

Additional levels of complexity in the role *S. aureus* plays in compromising PMN-fibrinogen interaction are possible by considering the presence of additional cell wall-associated and extracellular fibrinogen-binding proteins expressed by the bacteria. However, these proteins do not appear to play a major role in promoting bacterial attachment to immobilized fibrinogen. In addition, two of the better characterized proteins, Efb, an extracellular fibrinogen-binding protein (26) and, ClfB, a cell wall associated MSCRAMM related to ClfA (23), interact with the α-chain and with the α- and β-chains of fibrinogen, receptively. Thus, interference of PMN fibrinogen-binding activity, mediated by the γ-chain, is unlikely. It may be argued that ClfA inhibition of PMN adherence to fibrinogen would be more effective if the protein was extracellular and not bound to the bacterial cell wall.

However, it has been demonstrated that *S. aureus* binds effectively to endothelium via fibrinogen molecules (4), to fibrin blood clots and to fibrinogen-coated biomaterials (7), and the experiments in accordance with the present invention have shown that the ligand-binding regions of ClfA, namely Clf40 (40-559) and Clf41 (221-559), can inhibit the interaction and adhesion between polymorphonuclear leukocytes and immobilized fibrinogen. Accordingly, the present invention can be utilized in those applications, such as the development of vascular plaque or acute inflammation at the situs of a diseased blood vessel or one that has undergone vascular surgery, wherein reduction of the effects of such adhesion is highly desirable.

EXAMPLE 3: ADDITIONAL INVESTIGATIONS REGARDING PHAGOCYTOSIS OF *S. AUREUS*

Preparation of FITC-labeled bacteria.

The strains of *S. aureus* to be labeled were grown overnight without shaking at 37°C in Mueller-Hinton broth, harvested by centrifugation and washed once with 0.9% NaCl. Following resuspension in 0.1M $NaHCO_3$ (pH 9.0), bacterial cells were incubated with FITC Isomer I (100μg/ml) (Molecular Probes, Leiden, NL) in 0.5M $NaHCO_3$ buffer (pH 9.0), for 1 hour at 37°C. Following conjugation, the cells were washed and fixed with 0.5% (v/v) formaldehyde in PBS for 1 hour at room temperature. After one wash with PBS, the cells were resuspended in PBS with 1% BSA. Before use, the FITC-conjugated bacteria were stored at 4°C.

Phagocytosis of FITC-conjugated *S. aureus* by human PMN.

FITC-conjugated *S. aureus* strains (Newman wild-type, Newman Δ*clfA*, Newman Δ*spa* and Newman Δ*clfA* Δ*spa*) (1 x 10^7 cells/ml) were suspended in PBS with or without 10% normal human serum (NHS) from healthy donors. Following incubation for 30 min. at 37°C, the cells were washed twice in PBS with 1% BSA and resuspended in the same buffer. To ensure the cells were in suspension as single cells, the *S. aureus* cell suspension was sonicated gently and evaluated microscopically. A 500μl volume of FITC-labeled *S. aureus* suspension (1 x 10^7 cells/ml) was mixed with 500μl of purified human PMN isolated from whole blood from healthy donors (2 x 10^6 cells/ml) (1A). Following incubation for 0 or 5 min at 37°C, phagocytosis was terminated by placing the PMN-*S. aureus* cell mixture on ice. After 5 min, the cell suspension was centrifuged briefly and the pellet was resuspended in

500µl of PBS with 2.5% BSA for analysis by flow cytometry. To distinguish between bacteria adherent to PMN and bacteria internalized within PMN, trypan blue was added to quench fluorescence due to extracellular FITC-conjugated bacteria. As trypan blue cannot enter PMN cells, fluorescence due to phagocytosed bacteria was not affected by addition of the dye.

Flow cytometric analysis was performed on a Becton Dickinson FACScan cytometer with 488nm excitation light and emission collected through a 520/20nm bandpass filter. In general, 5000 cells were analyzed in the Fl-1 channel, and light scatter and fluorescence signals were collected with fixed amplification. The threshold settings for non-specific fluorescence were obtained by using PMN cells in the absence of FITC-labeled bacteria. Histograms of fluorescent FSC/SSC-gated cells were obtained. The level of phagocytosis (expressed as arbitrary fluorescence units (AFU)) was determined according to the following equation:

1AFU = (mean Fl-1 height of positive events (cells)) x (% of positive events (cells))/1000

Assessment of results.

S. aureus is a gram-positive bacterium, which is not sensitive to the lytic effect of complement but is attacked through the surface deposition of C3b. After initial deposition of the C3b molecule, the amplification system of the alternative pathway rapidly increases the deposition of C3b, making the bacteria sensitive to phagocytosis by phagocytic cells. The main C3b receptor on these cells is the integrin, (CR3). Additionally, coating of bacteria with serum immunoglobulins allows phagocytic cells to engulf the microorganism via the antibody Fc receptors on their surface.

We investigated the ability of two surface adhesins of *S. aureus*, the fibrinogen-binding protein, ClfA, and the IgG-binding protein, protein A, in protecting the bacteria from opsonophagocytosis by human PMNs. An earlier study implicated protein A in rendering *S. aureus* less susceptible to antibody-mediated phagocytosis (37). In this study using isogenic site-specific mutants of *S. aureus,* it was demonstrated that ClfA- and protein A-deficient strains were taken up more efficiently by PMNs compared to the wild-type strain (Fig. 6). A double mutant *S. aureus* strain in which both protein A and ClfA were deleted was dramatically more susceptible (by 200%) to phagocytosis by PMNs compared to the wild-type strain.

These data indicate that both protein A and ClfA contribute to protecting *S. aureus* from phagocytosis by human PMNs, and thus the use of these binding regions in accordance with the methods of the present invention may provide for enhanced bacterial phagocytosis. The finding that protein A inhibits PMN phagocytosis was not unexpected as protein A is known to interact with the Fc domain of IgG (38). As this region of the IgG molecule is also the binding site for the Fc receptors on PMNs, bound IgG on the surface of the bacteria will be unable to promote antibody-mediated phagocytosis. However, the discovery by the present inventors that ClfA interferes with phagocytosis is novel and has not been previously reported. The interaction of ClfA and fibrinogen could serve to coat the surface of the bacteria with fibrinogen and thus mask binding sites for opsonins present in normal sera, and ClfA may interact with iC3b by a mechanism similar to its interaction with $\alpha M\beta 2$.

In this study, it has been demonstrated that ClfA inhibits $\alpha M\beta 2$ fibrinogen-binding activity by interacting with, or in close proximity to, the $\alpha M\beta 2$-binding site in fibrinogen. The notion that ClfA shares functional similarities to was further strengthened when the ligand-binding region of ClfA was shown to have sequence

homology to an integrin-like protein (αInt1p) from *Candida albicans*, a protein which also shares functional and sequence homologies with αMβ2 (39). By interacting with iC3b at the αMβ2 binding site, ClfA could utilize molecular mimicry to inhibit complement-mediated phagocytosis.

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WHAT IS CLAIMED IS:

1. A method of inhibiting the adhesion of leukocytes to fibrinogen comprising administering an effective amount of a protein selected from the group consisting of Clf40 and Clf41 so that the protein inhibits said adhesion at a situs where inhibition of such adhesion is desired.

2. A method according to Claim 1 wherein the administration of the protein is directly at the situs wherein inhibition of leukocyte adhesion is desired.

3. A method according to Claim 1 wherein the administration of the protein is systemic and the protein will be systemically transmitted to the situs wherein inhibition of leukocyte adhesion is desired.

4. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are natural or recombinant proteins from staphylococcal bacteria.

5. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are natural or recombinant proteins from *Staphylococcus aureus*.

6. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are in the form of active fragments or portions of the natural or recombinant proteins.

7. A method according to Claim 1 wherein the protein is administered at the situs of a vascular injury.

8. A method according to Claim 1 wherein the protein is administered following a vascular surgical operation.

9. A method according to Claim 1 wherein the protein is administered at the situs of a vascular implant.

10. A composition for inhibiting the adhesion of leukocytes to fibrinogen comprising an isolated protein selected from the group consisting of Clf40 and Clf41 and a pharmaceutically acceptable vehicle, excipient or carrier.

11. An antibody raised against an isolated and purified form of a protein selected from the group consisting of Clf40 and Clf41.

12. A pharmaceutical composition comprising an antibody according to Claim 9 and a pharmaceutically acceptable vehicle, excipient or carrier.

13. A vaccine comprising an immunologically effective amount of a protein selected from the group consisting of Clf40 and Clf41 and a pharmaceutically acceptable carrier or excipient.

14. A vaccine according to Claim 13 wherein the proteins are from staphylococcal bacteria.

15. A vaccine according to Claim 13 wherein the proteins are from *Staphylococcus aureus*.

16. A vaccine according to Claim 13 wherein the proteins are in the form of immunogenic fragments or portions of the proteins.

17. A vaccine according to Claim 13 further comprising a pharmaceutically acceptable adjuvant.

18. A method of generating an immune response in a host comprising administering to said host an immunologically effective amount of the composition of Claim 10.

19. A method of enhancing the immunogenic response in a patient comprising administering to said patient an immunogenically effective amount of a pharmaceutical composition selected from the group consisting of a composition comprising an comprising a Clf40 or Clf41 protein and a pharmaceutically acceptable vehicle, excipient or carrier, and a composition comprising an antibody according to Claim 9 and a pharmaceutically acceptable vehicle, excipient or carrier.

20. A method of increasing phagocytosis in a patient comprising administering to said patient a pharmaceutical composition selected from the group consisting of a composition comprising an comprising a Clf40 or Clf41 protein and a pharmaceutically acceptable vehicle, excipient or carrier, and a composition comprising an antibody according to Claim 11 and a pharmaceutically acceptable vehicle, excipient or carrier, in an amount effective to increase phagocytosis in a patient.

21. A vaccine comprising a pharmaceutically acceptable formulation comprising nucleic acid encoding Clf40 or Clf41 and a pharmaceutically acceptable vehicle, carrier or excipient.

22. A kit for detecting Clf40 or Clf41 or antibodies thereto, comprising an isolated Clf40 or Clf41 protein and means to identify the binding thereto of an antibody in a sample suspected of containing such antibodies, or an antibody to an isolated Clf40 or Clf41 protein and means to identify the binding thereto of a Clf40 or Clf41 protein in a sample suspected of containing such proteins.

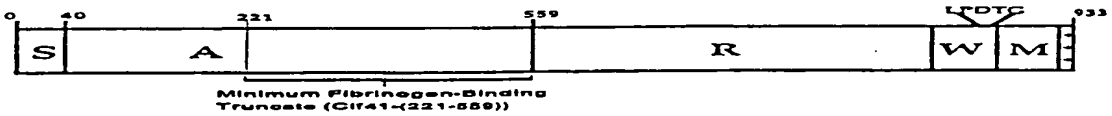


FIG. 1 Schematic model showing the domain organiztion of *S. aureus* clumping factor (ClfA). S, signal peptide; A, fibrinogen-binding region; R, repeat region; W, cell-wall spanning region; M, membrane-spanning domain; +, positively-charged tail. The minimum fibrinogen-binding domain is located between residues 221 and 559.

FIG. 2-
INSET

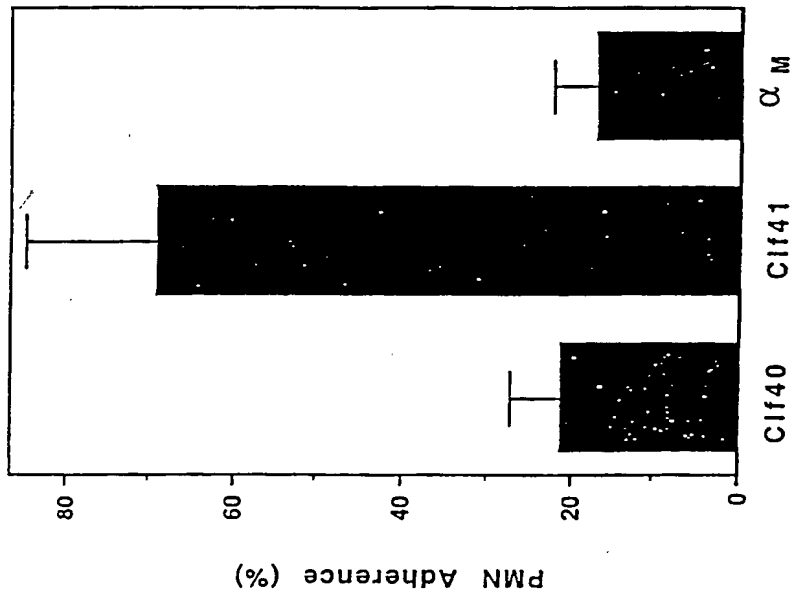


FIG. 2

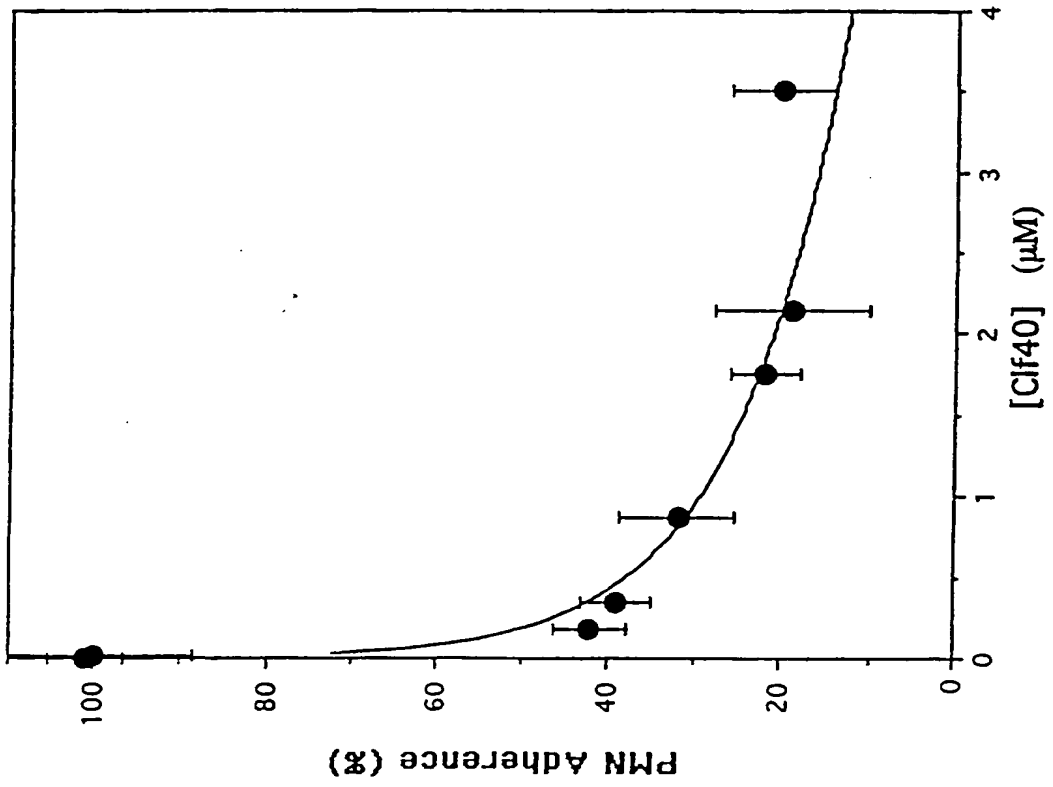
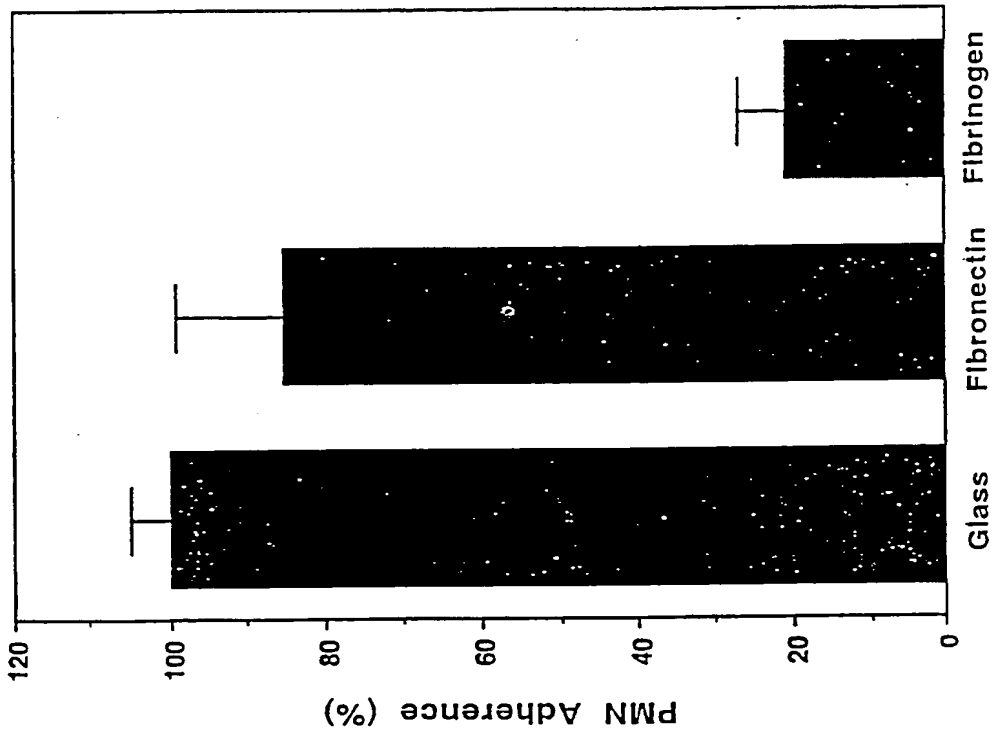


FIG. 3


PMN Adherence (%)
120
100
80
60
40
20
0
Glass
Fibronectin
Fibrinogen

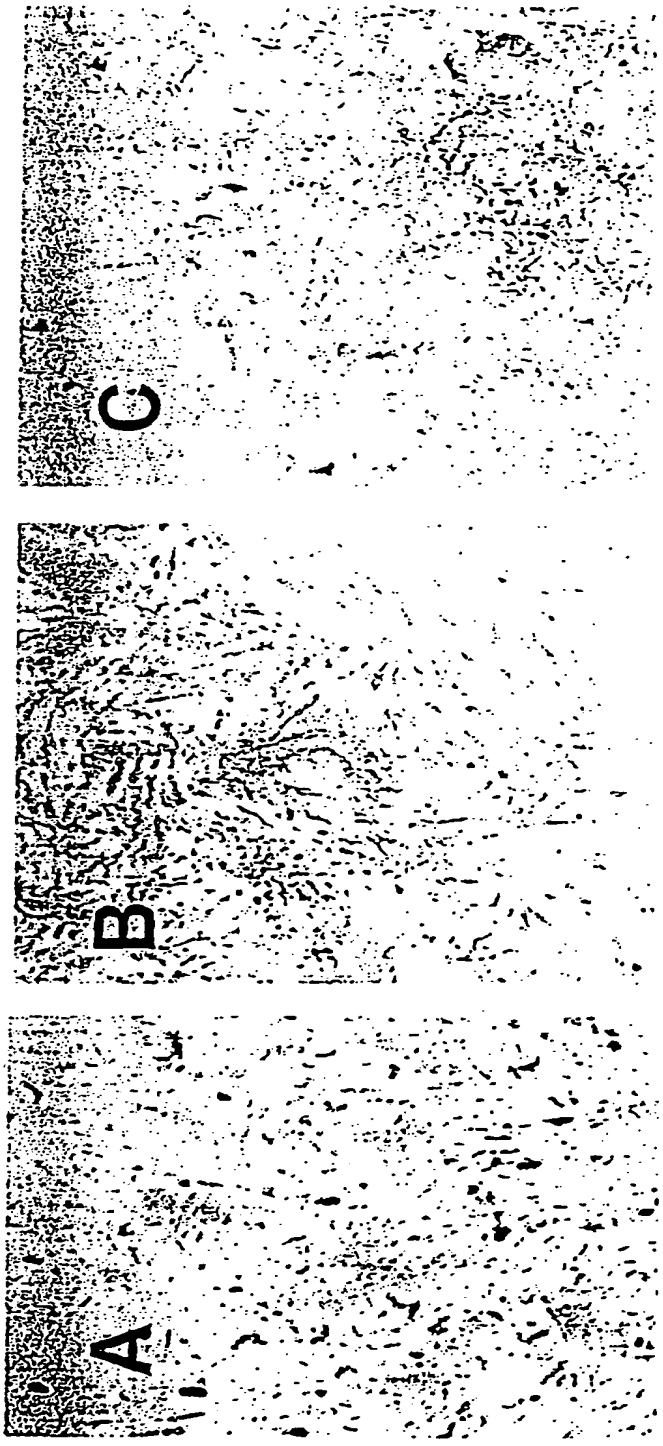


FIG. 4

FIG. 5

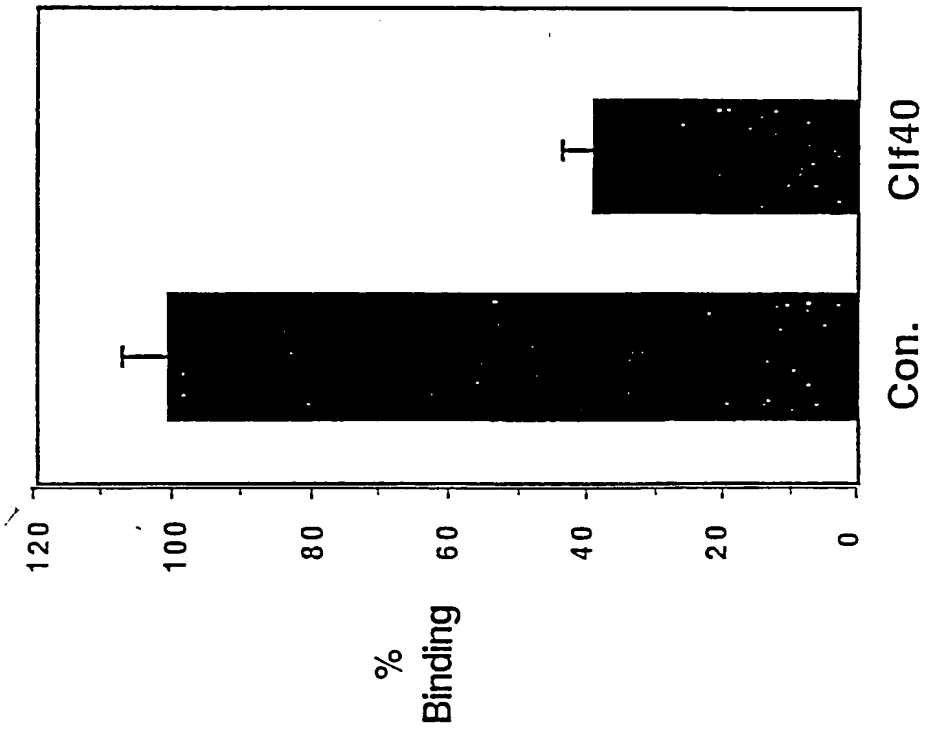
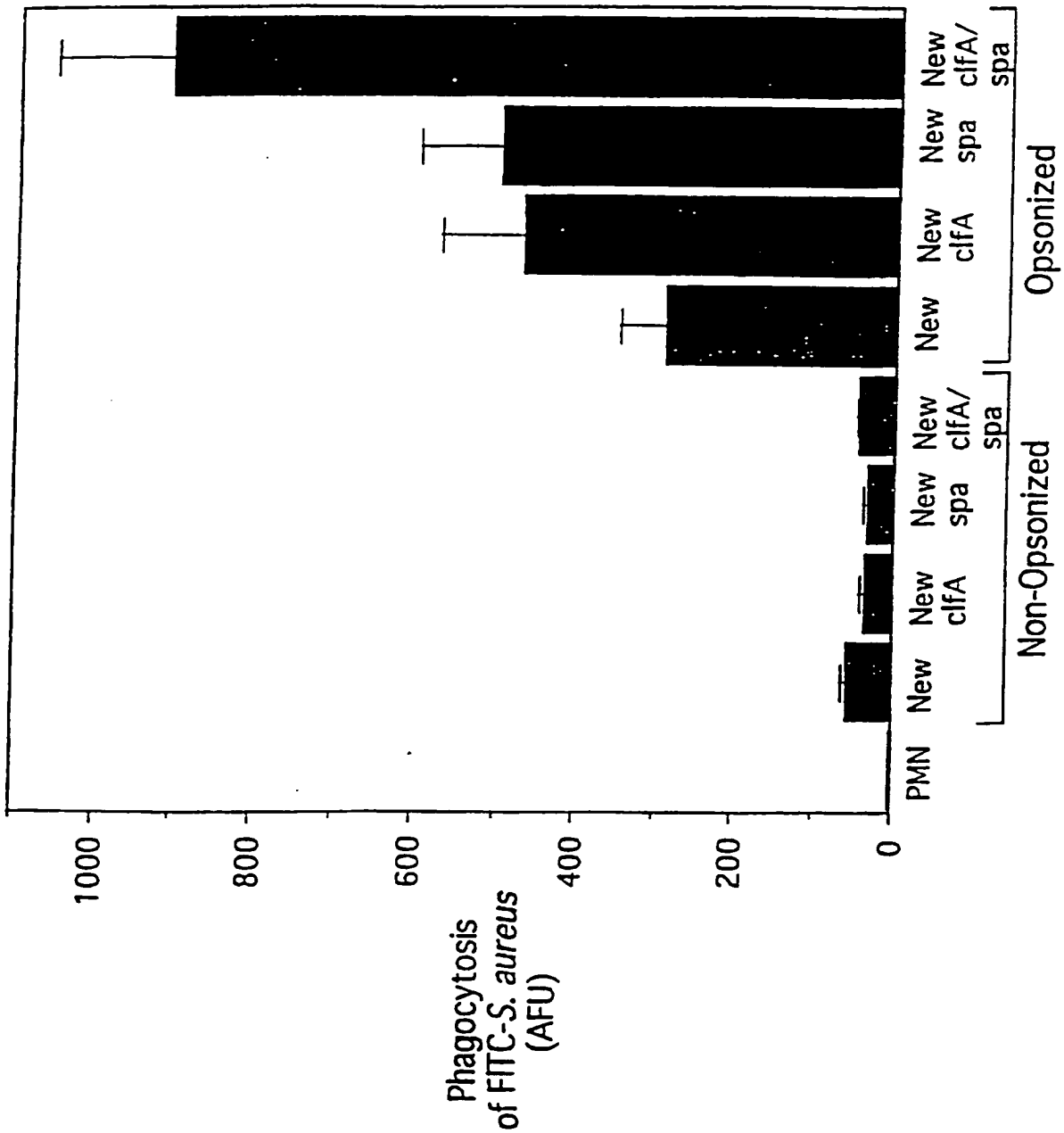


FIG. 6


Phagocytosis of FITC-S. aureus (AFU)
0
200
400
600
800
1000
PMN
New
New clfA
New spa
New clfA/ spa
New
New clfA
New spa
New clfA/ spa
Non-Opsonized
Opsonized

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11685

A. CLASSIFICATION OF SUBJECT MATTER

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US CL : 530/350; 536/23.7; 435/69.1, 69.3 252.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST: leukocyte adhesion, phagocytosis, fibrinogen, immunogenic response, clf40 and clf41 proteins, staphylococcus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,008,341 A (FOSTER et al.) 28 December 1999, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search 03 JULY 2000	Date of mailing of the international search report 11 AUG 2000
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(54) Title: METHOD OF INHIBITING LEUKOCYTE ADHESION TO FIBRINOGEN

(57) Abstract: A method is provided for reducing or preventing the adhesion of polymorphonuclear (PMN) leukocytes to fibrinogen such as might occur at the situs of a vascular injury due to disease or a surgical operation such as balloon angioplasty or a vascular transplantation by administration of a composition containing an effective amount of a Clf40 or Clf41 protein from the ligand-binding region of the ClfA protein from staphylococcal bacteria. The use of Clf40 or Clf41 compositions can inhibit adhesion to fibrinogen at the endothelial level so as to treat or prevent undesirable conditions associated with vascular injury, including the development of atherosclerotic plaque or inflammation. In addition, the present invention provides methods of isolating and purifying the Clf40 and Clf41 regions, as well as methods of using compositions containing these proteins in the treatment or prevention of infectious irritations or disease conditions caused by staphylococcal bacteria. Further, the invention contemplates the development and use of vaccines and antibodies based on the Clf40 and Clf41 protein regions, and the use of Clf40 and Clf41 in various methods including the generation of an immune response against these proteins, the enhancement of the immune response against staphylococcal bacteria, and the increase in the phagocytic capacity in the host to counter infection.

METHOD OF INHIBITING LEUKOCYTE ADHESION TO FIBRINOGEN

BACKGROUND OF THE INVENTION

(1) FIELD OF THE INVENTION

The present invention relates in general to a method of inhibiting leukocyte adhesion to fibrinogen, and in particular to the isolation and use of ligand-binding regions Clf40 and Clf41 from staphylococcal bacteria in methods of inhibiting or preventing the adhesion of polymorphonuclear leukocytes to endothelial tissue so as to inhibit the build up of neutrophils and prevent or reduce inflammation following vascular injury or vascular surgery. In addition, the present invention provides compositions based on the Clf40 or Clf41 regions and their use in generating or enhancing an immune response against staphylococcal bacteria.

(2) DESCRIPTION OF THE RELATED ART

Staphylococcal bacteria, such as *Staphylococcus aureus*, are important human pathogens that can cause diseases both in healthy individuals and in hospital patients. These range from superficial skin infections to life-threatening diseases including endocarditis, osteomyelitis and septic arthritis. Unfortunately, over the years, many traditional methods used to prevent or control the spread or the severity of disease caused by pathogenic staphylococcal bacterial, primarily antibiotic treatment regimens, are no longer effective. Accordingly, the medical community is constantly looking for other modes by which such infections and other diseases can be controlled.

More recently, it has been learned that the adherence of these bacteria to host matrix components, mediated by bacterial surface adhesins, is the initial

critical event in the pathogenesis of most infections. The extra-cellular matrix (ECM) of the host contains numerous macromolecules, including glycoproteins and proteoglycans, that support cellular adhesion and migration of host cells. These processes involve integrins, a family of heterodimeric cell-surface receptors that recognize specific ECM proteins. It has become increasingly evident that bacteria, including *S. aureus*, also utilize the ECM as substrata for their adhesion by way of a family of adhesins called MSCRAMM (microbial surface components recognizing adhesive matrix molecules)(8) that specifically recognize host matrix components.

One important component of the ECM, also occurring in soluble form in blood plasma, is fibrinogen. *S. aureus* possesses several fibrinogen-binding proteins, one of which (clumping factor, ClfA) is primarily responsible for bacterial adherence to fibrinogen substrata (17). ClfA is the prototype of a recently identified multigene family of putative surface proteins characterized by a common domain composed of a unique serine-aspartate repeat (10)(Fig. 1). The gene for the clumping factor protein ClfA, has recently been cloned, sequenced and analyzed in detail at the molecular level (17, 43). The predicted protein is composed of 933 amino acids. A signal sequence of 39 residues occurs at the N-terminus followed by a 520 residue region (region A), which contains the fibrinogen binding domain. A 308 residue region (region R), composed of 154 repeats of the dipeptide serine-aspartate, follows. The R region sequence is encoded by the 18 basepair repeat GAY TCN GAY TCN GAY AGY in which Y equals pyrimidines and N equals any base. The C-terminus of ClfA has features present in many surface proteins of gram-positive bacteria such as an LPDTG

motif, which is responsible for anchoring the protein to the cell wall, a membrane anchor, and positive charged residues at the extreme C-terminus. Additional information concerning fibrinogen and fibrinogen binding proteins is disclosed in co-pending applications, including Ser. Nos. 09/421,868, 09/386,960, 09/386,959, and 09/200,650, and in U.S. Pat. No. 6,008,341, and all of these applications and patent references are incorporated by reference.

Previously, we have shown that ClfA of *S. aureus* shares functional similarities with the platelet integrin, $\alpha_{IIB}\beta_3$. These similarities include the presence of calcium-binding EF-hands that inhibit ligand-binding function and also that both proteins interact with the extreme C-terminus of the fibrinogen γ-chain (18, 24). The notion that ClfA interacts with fibrinogen by a similar mechanism to integrins was strengthened when the ligand-binding region of ClfA was shown to have sequence homology to an integrin-like protein (αInt1p) from *Candida albicans* (9). This protein, which interacts with fibrinogen, also has amino acid sequence similarity with the I-domain of the fibrinogen-binding integrin, αMβ2(Mac-1/CR3), the major integrin of phagocytic cells including polymorphonuclear leukocytes (PMN).

Evidence is now emerging that the interaction of αMβ2 with fibrinogen is important for cell-cell interactions. The αMβ2-fibrinogen interaction has been directly implicated in leukocyte adhesive reactions during immune and inflammatory responses. The engagement of fibrinogen by αMβ2 on activated leukocytes and by ICAM-1 on endothelial cells mediates leukocyte adhesion to the vessel wall and subsequent transmigration into inflamed tissue (13, 14, 28).

The binding of fibrinogen/fibrin may also result in adhesion of monocytes and neutrophils at sites of vascular injury, such as atherosclerotic plaques (33). Furthermore, fibrinogen and its derivatives directly promote accumulation of inflammatory cells on bio-material implants in animal models and their depletion may abrogate this response (30, 31).

Recognition of fibrinogen by involves two sites within the γ-chain, γ191-202 (P1) and γ377-395 (P2) (1, 32). Although separated in linear amino acid sequence γ190-202 and γ377-395 are brought into close proximity by the folding of the γ-chain module (34). Peptide and structural analysis of the P2 binding site suggests that residues 383-395 represents a major recognition site (32). Key residues involved in recognition should be exposed on the surface of the γ-chain module of fibrinogen. Examination of the crystal structure reveals that the side-chains of the residues forming the β-strand, γ380-390, are buried or partially buried. However, amino acid residues positioned beyond this β-strand towards the extreme C-terminus of the γ-chain (γ390-395) are surface exposed and are particularly good candidates to play a critical role in αMβ2-binding activity. In addition, these residues are positioned on the face of the fibrinogen γ-chain module from where the extreme C-terminus, which is a flexible appendage in intact fibrinogen, emerges from the modular structure. Although the flexible nature of the fibrinogen γ-chain C-terminus and its close proximity to the major binding sites have been evidenced, it has not previously been shown that ClfA, by interacting with the γ-chain C-terminus, could come into contact with amino acid residues involved in fibrinogen-interaction or could effect exposure of the P1 and P2 binding sites on

the surface of intact fibrinogen via a conformational change. In either case, there exists a distinct need in the field to examine the interaction between ClfA and fibrinogen and utilize this interaction to potentially interfere with fibrinogen-binding activity.

As indicated above, the binding of fibrinogen/fibrin may result in adhesion of leukocytes and neutrophils at sites of vascular injury, and thus may give rise to a detrimental conditions such as the development of atherosclerotic plaques (33). In addition, fibrinogen and its derivatives are believed to directly promote the accumulation of inflammatory cells on surfaces of biomaterial implants in animal models (30, 31) and at sites of vascular injury, which again may need to be treated and/or alleviated. This has been a particular problem in vascular surgical procedures such as balloon angioplasty wherein vascular injury in the coronary artery leads to neutrophil and platelet activation on plaque following such a procedure. For example, it has been shown (40) that activation of blood cells at the balloon-injured coronary artery plaque occurs, and that neutrophil and platelet activation at the situs of the balloon-injury plaque contributes to abrupt vessel closure and late restenosis after percutaneous transluminal coronary angioplasty.

Moreover, although attempts have been made to counteract the buildup of plaque due to this activation following disease or surgical injury to vascular tissue using antibodies (41, 42), there is still a serious need to develop methods of reducing or preventing the interaction of leukocytes, such as polymorphonuclear leukocytes, with immobilized fibrinogen so as to prevent or alleviate conditions such as plaque development or inflammation at the situs of a vascular injury.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide compositions comprised of purified ligand-binding regions of ClfA, such as Clf40 and Clf41, which can be used in methods of reducing or preventing PMN leukocyte adhesion to fibrinogen which might otherwise lead to undesirable conditions such as the development of vascular plaque or deleterious inflammation at the situs of a vascular injury caused by disease or a surgical operation, such as a balloon angioplasty or other vascular surgery, or following a vascular transplantation or implantation procedure.

It is a further object of the present invention to provide isolated and purified peptide compositions of the Clf40 or Clf41 domains that are useful in methods of inhibiting adhesion of ClfA or PMN leukocytes to fibrinogen present on the surface of host cells or implanted biomaterials.

It is a further object of the present invention to provide a vaccine based on ligand-binding regions of the fibrinogen-binding domain, such as Clf40 or Clf41, which can be used in treating or preventing infection by staphylococcal bacterial such as *Staphylococcus aureus*, and which can be utilized in methods of enhancing the immune response to staphylococcal infection and increasing phagocytosis.

It is still further an object of the present invention to generate antisera and antibodies to the purified ligand-binding regions of ClfA, such as Clf40 and Clf41, which can be useful in methods of inhibiting staphylococcal adhesion to fibrinogen in general and which can inhibit staphylococcal adhesion to fibrinogen interacting with an endothelial monolayer.

These and other objects are provided by virtue of the present invention which comprises methods of isolating the ligand-binding region of ClfA such as Clf40 and Clf41 which can inhibit adhesion to fibrinogen of PMN leukocytes such as may occur at endothelial monolayers so as to treat or prevent undesirable conditions such as the development of vascular plaque or deleterious inflammation at the situs of a vascular injury, such as may result from disease or a surgical operation, e.g., balloon angioplasty. In addition, the present invention provides methods of isolating and purifying the Clf40 and Clf41 regions, as well as methods of using compositions containing these proteins in the treatment or prevention of infectious irritations or disease conditions caused by staphylococcal bacteria, including those associated with fibrinogen binding. Further, the invention contemplates the development and use of vaccines and antibodies based on the Clf40 and Clf41 protein regions, and the use of Clf40 and Clf41 in various methods including the generation of an immune response against these proteins, the enhancement of the immune response against staphylococcal bacteria, and the increase in the phagocytic capacity in the host to counter infection.

These and other objects of the present invention will be described more fully in, or will become obvious from, the detailed description of the preferred embodiments provided hereinbelow.

BRIEF DESCRIPTION OF THE DRAWING FIGURES

FIG. 1 depicts a schematic model showing the domain organization of *S. aureus* clumping factor (ClfA) wherein S is a signal peptide; A, fibrinogen-binding

region; R, repeat region; W, cell-wall spanning region; M, membrane-spanning domain; +, positively-charged tail. The minimum fibrinogen-binding domain is located between residues 221 and 559.

FIG. 2 (and inset FIG. 2A) depicts the inhibition of PMN adherence to immobilized fibrinogen with increasing concentrations of recombinant Clf40-(40-559). Immobilized fibrinogen on glass was pre-incubated with recombinant protein for 1 hr. at 37°C. Unbound protein was removed by washing with PBS. PMN (5 x 10^5 cells) were incubated with immobilized fibrinogen for 20 min. at 37°C. Adherent PMN were quantified microscopically. The number of PMN binding to fibrinogen in the absence of inhibitors was assigned a value of 100%.

FIG. 2A, the *Inset,* depicts the inhibition of PMN (5 x 10^5 cells) adhesion to immobilized fibrinogen by recombinant Clf40-(40-559) (3.5μM), Clf41-(221-559) (5.2μM) and α_M I-domain (3.5μM). Data are expressed as means ± SD (error bars) (*n*=4).

FIG. 3 depicts the inhibition of PMN adherence to glass, immobilized fibronectin and immobilized fibrinogen with recombinant Clf40-(40-559) (3.5μM). Adherent PMN were quantified microscopically. The number of PMN attached to each substrate in the absence of Clf40-(40-559) was assigned a value of 100%. Data are expressed as means ± SD (error bars) (*n* = 4).

FIG. 4 depicts the adherence of PMN to an human umbilical vein endothelial cell monolayer. PMN (5 x 10^6 cells) were added to immobilized endothelial cells that had been pre-incubated with fibrinogen (1 mg/ml) (A), fibrinogen (1 mg/ml) and Clf40-(40-559) (23.7μM) (B), and Clf40-(40-559)

(23.7μM) in the absence of fibrinogen (C). After washing with PBS enriched in Ca^{2+} and Mg^{2+} and fixing in 4% paraformaldehyde in PBS, adherent PMN granules were stained with the benzidine hydrochloride-based Kaplow stain (12). The degree of cell attachment was evaluated microscopically.

FIG. 5 depicts the inhibition of the interaction between biotinylated α_M I-domain and immobilized fibrinogen (5μg/ml) by Clf40-(40-559). Bound protein was detected with alkaline phosphatase-labeled avidin and quantitated colorimetrically. Binding of α_M I-domain in absence of Clf40-(40-559) was assigned a value of 100. Data are expressed as means ± SD (error bars) (n = 3).

FIG. 6 depicts the phagocytosis of FITC-*S. aureus* strains by purified human polymorphonuclear leukocytes. *S. aureus* wild-type and mutants were pretreated with 0% NHS (Non-opsonized) or 10% NHS (Opsonized) and then incubated with human PMNs (1×10^6 cells) for 5 minutes at 37°C. The level of phagocytosis of each strain of *S. aureus* by PMNs was determined by flow cytometry as described in the Method section. Results are expressed as mean ± standard error of the mean (n = 5).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, there is provided a method of preventing adhesion of leukocytes, such as polymorphonuclear (or PMN) leukocytes, to immobilized fibrinogen which comprises administering an effective amount of a composition comprising a Clf40 or Clf41 protein to a human or animal patient in such a manner that it will prevent or reduce the effects, such as

development of plaque or inflammation, caused or aggravated by the adhesion of leukocytes to fibrinogen. This may be accomplished in a variety of ways including administration of one or more of these proteins directly at the situs of the injury or systemically in a manner where the proteins will ultimately work at the desired location. As would be recognized by one of ordinary skill in this art, the Clf40 or Clf41 proteins as utilized in the present invention would encompass active fragments or portions thereof which effect the inhibition of leukocyte adhesion to fibrinogen in the same manner as the protein in its complete form.

As discussed above, it has been shown that the binding of fibrinogen/fibrin may result in adhesion of leukocytes and neutrophils at sites of vascular injury, and thus may give rise to a detrimental conditions such as the development of atherosclerotic plaques (33). In addition, fibrinogen and its derivatives are believed to directly promote the accumulation of inflammatory cells on surfaces of biomaterial implants in animal models (30, 31) and at sites of vascular injury, which again may need to be treated and/or alleviated. This problem has been particularly observed with regard to vascular surgical procedures such as balloon angioplasty wherein vascular injury in the coronary artery leads to neutrophil and platelet activation on plaque following such a procedure. In fact, it has been shown (40) that activation of blood cells at balloon-injured coronary artery plaque occurs, and that neutrophil and platelet activation at the situs of the balloon-injury plaque contributes to abrupt vessel closure and late restenosis after percutaneous transluminal coronary angioplasty. Accordingly, the method of the present invention will thus be useful in preventing or alleviating such conditions by achieving the inhibition of adhesion of leukocytes, such as polymorphonuclear (or

PMN) leukocytes, to immobilized fibrinogen wherever necessary to treat or prevent these deleterious vascular conditions.

Further, the Clf40 and Clf41 proteins of the present invention may be useful as analogs in the development and/or identification of small molecules which can mimic the ability of Clf40 and Clf41 and thus be utilized in methods for treating or preventing deleterious conditions associated with the adhesion of leukocytes to fibrinogen. In this case, Clf40 and Clf41 proteins may be used to determine more precisely the relevant ClfA binding sites and the relevant peptide sequences involved in such binding so as to allow one to develop smaller molecules which contain these active regions.

In addition, the present invention will be suitable for inhibiting adhesion of leukocytes on biological implants such as those implants utilized in transplants of natural or artificial blood vessels or other organs. Accordingly, the leukocyte adhesion-inhibiting compositions of the present invention will be useful in numerous applications wherein such effects are desired. For example, medical devices or polymeric biomaterials to be treated with the leukocyte adhesion-inhibiting compositions of the present invention may include, but are not limited to, staples, sutures, replacement heart valves, cardiac assist devices, hard and soft contact lenses, intraocular lens implants (anterior chamber or posterior chamber), other implants such as corneal inlays, kerato-prostheses, vascular stents, epikeratophalia devices, glaucoma shunts, retinal staples, scleral buckles, dental prostheses, thyroplastic devices, laryngoplastic devices, vascular grafts, soft and hard tissue prostheses including, but not limited to, pumps, electrical devices including stimulators and recorders, auditory prostheses, pacemakers, artificial

larynx, dental implants, mammary implants, penile implants, cranio/facial tendons, artificial joints, tendons, ligaments, menisci, and disks, artificial bones, artificial organs including artificial pancreas, artificial hearts, artificial limbs, and heart valves; stents, wires, guide wires, intravenous and central venous catheters, laser and balloon angioplasty devices, vascular and heart devices (tubes, catheters, balloons), ventricular assists, blood dialysis components, blood oxygenators, urethral/ureteral/urinary devices (Foley catheters, stents, tubes and balloons), airway catheters (endotracheal and tracheostomy tubes and cuffs), enteral feeding tubes (including nasogastric, intragastric and jejunal tubes), wound drainage tubes, tubes used to drain the body cavities such as the pleural, peritoneal, cranial, and pericardial cavities, blood bags, test tubes, blood collection tubes, vacutainers, syringes, needles, pipettes, pipette tips, and blood tubing.

As will be recognized by one skilled in the art, because of the numerous possible applications of the methods of the present invention, and because the inhibition of leukocyte adhesion by Clf40 is dose-dependent, as described further below, one skilled in the art will recognize that any particular treatment regimen will have to be determined on the basis of the extent and nature of the condition to be treated, and that the amount of the particular protein administered will fall within a range of values which would be suited to treat the particular condition as needed for the particular application. As indicated above, these conditions primarily include vascular or endothelial injury associated with a variety of pathogenic diseases or a variety of surgical operations ranging from angioplasty

and other similar operations to transplantation and the implantation of vascular prosthetic devices.

As would be recognized by one skilled in the art, the Clf40 and Clf41 proteins of the present invention, or active fragments thereof, may be prepared in a number of conventional ways, including isolation and purification of the natural proteins, or more preferably, using recombinant methods well known in the art to produce isolated and purified forms of these proteins. For example, in a particularly preferred embodiment, the ClfA protein compositions of the present invention may be prepared using a bacterial host such as *Escherichia coli* XL-1 Blue for plasmid cloning and protein expression, growing the plasmid-containing bacterial host in suitable nutrient media containing desired additives such as Ampicillin. Next, expression plasmids may be cloned in a suitable manner known in the art, which in the preferred embodiment comprises using amplified fragments of the *ClfA* gene cloned into an expression plasmid such as pQE30 (Qiagen Inc.) to generate the constructs. In accordance with the present invention, the ligand-binding regions Clf40 and Clf41 are preferably prepared using plasmids pCF40-(40-559) and pCF41-(221-559), respectively, as has been described previously in references (see, e.g., Ref no. 24) which are incorporated herein. The recombinant protein expressed from these vectors contains an N-terminal extension of six histidine residues.

The recombinant proteins of the present invention can then be expressed and purified once again using conventional techniques well known in the art, such as by expressing of the plasmids in a bacterial host followed by purification in an appropriate chromatographic apparatus. In the preferred embodiment, the

recombinant Clf40-(40-559) of the invention can be expressed in a bacterial host such as *E. coli* XL-1 Blue and purified by immobilized metal chelate affinity chromatography and ion-exchange chromatography as described previously (24). Analysis by sodium dodecyl sulfate-polyacrylamide electrophoresis with Coomassie blue staining and Western immunoblotting indicated that a single immunoreactive protein of the correct molecular weight was obtained. In addition, the recombinant Clf41-(221-559) can be expressed and purified in the same way. For testing purposes, the glutathione S-transferase-α_M I-domain fusion protein was purified by glutathione-Sepharose (Pharmacia Biotech Inc.) affinity chromatography and cleaved with bovine thrombin according to manufacturers instructions.

In the preferred embodiment, the Clf40 or Clf41 proteins of the present invention, or active portions or fragments thereof, as well as antibodies as will be discussed further below, may be formulated in combination with a suitable pharmaceutical vehicle, excipient or carrier such as would be well known in this art. Examples of such suitable pharmaceutical vehicles, excipients or carriers include saline, dextrose, water, glycerol, ethanol, other therapeutic compounds, and combinations thereof. The formulation should be appropriate for the mode of administration, and should be compatible with the preferable use of the compositions of the invention, namely methods of treatment of conditions associated with vascular disease or surgical injury such as inflammation and the development of atherosclerotic plaque.

In addition to the use of the isolated, recombinant or synthetic proteins of the present invention, or antigenic portions thereof (including epitope-bearing fragments), or fusion proteins including the Clf40 or Clf41 proteins in the therapeutic leukocyte adhesion-inhibiting manner as described above, the proteins, or antigenic portions thereof, can be utilized to generate antibodies, which can then be isolated, purified and/or further utilized or administered for therapeutic purposes or for detection and determination of staphylococcal infections. Accordingly, in accordance with the invention, there is provided a method of generating an immune response to the Clf40 and Clf41 proteins using conventional means well known in this field to generate such a response. For example, an immune response may be produced when the immunogen (e.g., Clf40, Clf41 or an immunogenic fragment or portion thereof) is injected into humans or animals, including mice, rabbits, rats, goats, sheep, guinea pigs, chickens, and other animals.

Further, to enhance immunogenicity, the proteins may be conjugated to a carrier molecule. Suitable immunogenic carriers include proteins, polypeptides or peptides such as albumin, hemocyanin, thyroglobulin and derivatives thereof, particularly bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), polysaccharides, carbohydrates, polymers, and solid phases. Other protein derived or non-protein derived substances are known to those skilled in the art. An immunogenic carrier typically has a molecular weight of at least 1,000 Daltons, preferably greater than 10,000 Daltons. Carrier molecules often contain a reactive group to facilitate covalent conjugation to the hapten. The carboxylic acid group or amine group of amino acids or the sugar groups of glycoproteins

are often used in this manner. Carriers lacking such groups can often be reacted with an appropriate chemical to produce them. Alternatively, a multiple antigenic peptide comprising multiple copies of the protein or polypeptide, or an antigenically or immunologically equivalent polypeptide may be sufficiently antigenic to improve immunogenicity without the use of a carrier.

The Clf40 or Clf41 proteins (which encompasses or immunogenic fragments or portions thereof) or appropriate combination of proteins, may also be administered with an adjuvant in an amount effective to enhance the immunogenic response against the conjugate. For example, one such adjuvant widely used in humans has been alum (aluminum phosphate or aluminum hydroxide). Saponin and its purified component Quil A, Freund's complete adjuvant and other adjuvants used in research and veterinary applications may also be employed, but these adjuvants have toxicities which limit their potential use in human vaccines. However, suitable chemically defined preparations include muramyl dipeptide, monophosphoryl lipid A, phospholipid conjugates or encapsulation of the conjugate within a proteoliposome (such as those described in References 44 and 45, below, and incorporated herein by reference), and encapsulation of the protein in lipid vesicles such as Novasome™ lipid vesicles (Micro Vescular Systems, Inc., Nashua, NH) may also be useful.

In addition, the present invention may be utilized as immunological compositions, including vaccines, and other pharmaceutical compositions containing the Clf40 or Clf41 proteins or portions thereof are included within the scope of the present invention. Either one or both of the Clf40 or Clf41 proteins, or active or antigenic fragments thereof, or fusion proteins thereof, can be

formulated and packaged, alone or in combination with other antigens, using methods and materials known to those skilled in the art for vaccines. The immunological response may be used therapeutically or prophylactically and may provide antibody immunity or cellular immunity, such as that produced by T lymphocytes.

The term "vaccine" as used herein includes vaccines prepared from the Clf40 or Clf41 compositions of the present invention as well as DNA vaccines in which the nucleic acid molecule encoding the ligand-binding regions of the present invention is used in a pharmaceutical composition is administered to a patient. For genetic immunization, suitable delivery methods known to those skilled in the art include direct injection of plasmid DNA into muscles, delivery of DNA complexed with specific protein carriers, coprecipitation of DNA with calcium phosphate, encapsulation of DNA in liposomes, particle bombardment, and *in vivo* infection using cloned retroviral vectors.

In a preferred embodiment, a vaccine is packaged in a single dosage for immunization by parenteral (i.e., intramuscular, intradermal or subcutaneous) administration or nasopharyngeal (i.e., intranasal) administration. The vaccine is most preferably injected intramuscularly into the deltoid muscle. The vaccine is preferably combined with a pharmaceutically acceptable carrier to facilitate administration. The carrier is usually water or a buffered saline, with or without a preservative. The vaccine may be lyophilized for resuspension at the time of administration or in solution. The vaccine may additionally contain stabilizers or pharmaceutically acceptable preservatives, such as thimerosal (ethyl(2-

mercaptobenzoate-S)mercury sodium salt) (Sigma Chemical Company, St. Louis, MO).

The immunological compositions, such as vaccines, and other pharmaceutical compositions can be used alone or in combination with other blocking agents as appropriate to protect against human and animal infections caused by or exacerbated by staphylococci. The compositions thus may protect humans or ruminants against various staphylococcal infections, and the vaccine may also be useful in protecting other species of animals, for example canine and equine animals, against similar staphylococcal infections.

In accordance with the invention, the antibodies to Clf40 or Clf41, or to fragments thereof, such as described above, can also be used for the specific detection of fibrinogen-binding staphylococcal proteins, for the prevention of infection from staphylococci, for the treatment of an ongoing infection, or for use as research tools. The term "antibodies" as used herein includes monoclonal, polyclonal, chimeric, single chain, bispecific, simianized, and humanized or primatized antibodies as well as Fab fragments, including the products of an Fab immunoglobulin expression library. Generation of any of these types of antibodies or antibody fragments is well known to those skilled in the art. In the present case, specific polyclonal antiserum utilizing the proteins of the present invention may be generated and used for specific agglutination assays to detect bacteria which express the proteins of the present invention.

Additionally, any of the above described antibodies may be labeled directly with a detectable label for identification and quantification of staphylococci. Labels for use in immunoassays are generally known to those skilled in the art

and include enzymes, radioisotopes, and fluorescent, luminescent and chromogenic substances, including colored particles such as colloidal gold or latex beads. Suitable immunoassays include enzyme-linked immunosorbent assays (ELISA).

Alternatively, the antibody may be labeled indirectly by reaction with labeled substances that have an affinity for immunoglobulin. The antibody may be conjugated with a second substance and detected with a labeled third substance having an affinity for the second substance conjugated to the antibody. For example, the antibody may be conjugated to biotin and the antibody-biotin conjugate detected using labeled avidin or streptavidin. Similarly, the antibody may be conjugated to a hapten and the antibody-hapten conjugate detected using labeled anti-hapten antibody. These and other methods of labeling antibodies and assay conjugates are well known to those skilled in the art. Antibodies to the fibrinogen-binding proteins Clf40 or Clf41, or portions thereof, may also be used in production facilities or laboratories to isolate additional quantities of the proteins, such as by affinity chromatography.

In addition to the methods described above, it is also the case that the interaction of leukocytes such as PMN with immobilized fibrinogen plays an important role in the host immune response against pathogenic bacteria, and thus the present invention can be utilized in methods of enhancing the immune response against such infectious bacteria. As indicated above, this interaction is important in the inflammatory response because of the contribution of leukocyte adhesion to the endothelium and subsequent transmigration to sites of infection (see, e.g., references 13, 14, and 28 below). In order for PMN to initiate a

respiratory burst in response to soluble cytokines, they are required to utilize their β2 integrins (including αMβ2) and interact with immobilized matrix molecules such as fibrinogen (22). Furthermore, occupancy of αMβ2 with immobilized fibrinogen modulates monocyte oxidative response and phagocytosis of opsonized particles (31). Accordingly, the Clf40 and Clf41 protein compositions of the present invention may be utilized as appropriate by causing an enhancement in the immune response against staphylococcal bacteria, and in addition may promote opsonization and phagocytosis of staphylococcal bacteria in the host. In such methods, an amount of an appropriate composition in accordance with the invention, such as an antibody composition comprising antibodies generated to Clf40 or Clf41 in an amount effective to promote opsonization and phagocytosis of staphylococcal bacteria, or a Clf40 or Clf41 protein composition sufficient to generate an immune response thereto so as to enhance the immunogenic response against staphylococcal bacteria, is effectively administered to the patient.

Further, the Clf40 and Clf41 proteins and compositions in accordance with the present invention can be used to screen antibodies or antisera for hyperimmune patients from whom can be derived specific antibodies having a very high affinity for the proteins. Further, the present invention contemplates use in the detection of the presence of Clf40 or Clf41 or their antibodies and the diagnosis of related staphylococcal diseases ranging from superficial skin infections to life-threatening diseases including endocarditis, osteomyelitis and septic arthritis. In accordance with the invention, a preferred method of detecting

the presence of Clf40 or Clf41 proteins involves the steps of obtaining a sample suspected of containing staphylococci. The sample may be taken from an individual, for example, from one's blood, saliva, tissues, bone, muscle, cartilage, or skin, and the sample may be screened for the presence of Clf40, Clf41 or associated proteins or fragments using the compositions described above via suitable methods well known in the art.

The invention further contemplates a kit containing one or more Clf40 or Clf41-specific nucleic acid probes, which can be used for the detection of fibrinogen-binding proteins from staphylococci in a sample, or for the diagnosis of staphylococcal bacterial infections. A kit for the detection and/or diagnosis of staphylococcal infection is thus provided in accordance with the invention which comprises a means to identify the presence of Clf40 or Clf41 proteins in the sample, e.g., an antibody for Clf40 or Clf41 as described above, and appropriate means such as a label by which the sample can be detected is binding of the antibody occurs. Such a kit can also contain the appropriate reagents for hybridizing the probe to the sample and detecting bound probe. In an alternative embodiment, the kit contains antibodies specific to either or both Clf40 and Clf41 proteins or active portions thereof which can be used for the detection of staphylococci.

In yet another embodiment, the kit contains either or both the Clf40 and Clf41 proteins, or active fragments thereof, which can be used for the detection of staphylococcal bacteria or for the presence of antibodies to fibrinogen-binding staphylococcal proteins in a sample. The kits described herein may additionally contain equipment for safely obtaining the sample, a vessel for containing the

reagents, a timing means, a buffer for diluting the sample, and a colorimeter, reflectometer, or standard against which a color change may be measured.

In a preferred embodiment, the reagents, including the protein or antibody, are lyophilized, most preferably in a single vessel. Addition of aqueous sample to the vessel results in solubilization of the lyophilized reagents, causing them to react. Most preferably, the reagents are sequentially lyophilized in a single container, in accordance with methods well known to those skilled in the art that minimize reaction by the reagents prior to addition of the sample.

The isolated proteins of the present invention, or active fragments thereof, and antibodies to the proteins may be useful for the treatment and diagnosis of staphylococcal bacterial infections as described above, or for the development of anti-staphylococcal vaccines for active or passive immunization. Further, when administered as pharmaceutical composition to a situs where a vascular injury has occurred, whether from pathogenic disease, surgical operation or transplantation, the protein compositions of the present invention will be useful in inhibiting adhesion of leukocytes to the fibrinogen at that site which may be helpful in avoiding the development of atherosclerotic plaques or reducing deleterious inflammation.

When labeled with a detectable biomolecule or chemical, the fibrinogen-binding proteins described herein are useful for purposes such as *in vivo* and *in vitro* diagnosis of staphylococcal infections or detection of staphylococcal bacteria. Laboratory research may also be facilitated through use of such protein-label conjugates. Various types of labels and methods of conjugating the labels to the proteins are well known to those skilled in the art. Several specific labels

are set forth below. The labels are particularly useful when conjugated to a protein such as an antibody or receptor. For example, the protein can be conjugated to a radiolabel such as, but not restricted to, ^{32}P, ^{3}H, ^{14}C, ^{35}S, ^{125}I, or ^{131}I. Detection of a label can be by methods such as scintillation counting, gamma ray spectrometry or autoradiography.

Bioluminescent labels, such as derivatives of firefly luciferin, are also useful. The bioluminescent substance is covalently bound to the protein by conventional methods, and the labeled protein is detected when an enzyme, such as luciferase, catalyzes a reaction with ATP causing the bioluminescent molecule to emit photons of light. Fluorogens may also be used to label proteins. Examples of fluorogens include fluorescein and derivatives, phycoerythrin, allo-phycocyanin, phycocyanin, rhodamine, and Texas Red. The fluorogens are generally detected by a fluorescence detector.

The protein can alternatively be labeled with a chromogen to provide an enzyme or affinity label. For example, the protein can be biotinylated so that it can be utilized in a biotin-avidin reaction, which may also be coupled to a label such as an enzyme or fluorogen. For example, the protein can be labeled with peroxidase, alkaline phosphatase or other enzymes giving a chromogenic or fluorogenic reaction upon addition of substrate. Additives such as 5-amino-2,3-dihydro-1,4-phthalazinedione (also known as Luminol®) (Sigma Chemical Company, St. Louis, MO) and rate enhancers such as p-hydroxybiphenyl (also known as p-phenylphenol) (Sigma Chemical Company, St. Louis, MO) can be used to amplify enzymes such as horseradish peroxidase through a luminescent reaction; and luminogeneic or fluorogenic dioxetane

derivatives of enzyme substrates can also be used. Such labels can be detected using enzyme-linked immunoassays (ELISA) or by detecting a color change with the aid of a spectrophotometer. In addition, proteins may be labeled with colloidal gold for use in immunoelectron microscopy in accordance with methods well known to those skilled in the art. The location of a ligand in cells can be determined by labeling an antibody as described above and detecting the label in accordance with methods well known to those skilled in the art, such as immunofluorescence microscopy.

The following examples are provided which relate to various aspects of the development and use of the preferred embodiments of the present invention, and it should be appreciated by those of skill in the art that the techniques disclosed above and in the examples below are merely exemplary of the compositions and methods of the present invention. Accordingly, one of ordinary skill in this art will recognize that numerous alterations and amendments may be made to the embodiments disclosed in the present application which will still be considered to fall within the scope of the present invention.

EXAMPLES

EXAMPLE 1: PREPARATION OF CLFA PROTEIN COMPOSITIONS AND ASSAYS TO DETERMINE THEIR EFFECT ON PMN ADHESION TO FIBRINOGEN

Bacterial strains and growth conditions.

Escherichia coli XL-1 Blue (3) was used as the bacterial host for plasmid cloning and protein expression. *E. coli* cells harboring plasmids were routinely

grown in L-broth, Terrific broth, and L-agar (27). Ampicillin (100 μg/ml) was incorporated as appropriate.

Construction of expression plasmids.

Amplified fragments of the *ClfA* gene were cloned into the expression plasmid pQE30 (Qiagen Inc.) to generate the constructs pCF40-(40-559) and pCF41-(221-559) as described previously (24). Recombinant protein expressed from this vector contains an N-terminal extension of six histidine residues.

Expression and purification of recombinant proteins.

Recombinant Clf40-(40-559) was expressed in *E. coli* XL-1 Blue and purified by immobilized metal chelate affinity chromatography and ion-exchange chromatography as described previously (24). Analysis by sodium dodecyl sulfate-polyacrylamide electrophoresis with Coomassie blue staining and Western immunoblotting indicated that a single immunoreactive protein of the correct molecular weight was obtained (data not shown). Recombinant Clf41-(221-559) was expressed and purified in the same way. The glutathione S-transferase-α_M I-domain fusion protein was purified by glutathione-Sepharose (Pharmacia Biotech Inc.) affinity chromatography and cleaved with bovine thrombin according to manufacturers instructions.

PMN adherence assay.

Measurement of the adhesion of PMN to immobilized fibrinogen was carried out as described previously with minor modification (16). 100µl volumes were used for all additions. Briefly, glass slides were coated with purified fibrinogen (200µg/ml) for 1 h at 37°C in a moist chamber. Unbound fibrinogen was removed by washing in PBS and slides were post-coated with 1% human serum albumin for 2 h at 37°C. Purified human PMN isolated from whole blood from healthy donors (5 x 10^5 cells/ml in medium 199) (2) were allowed to adhere to immobilized fibrinogen for 20 min at 37°C. This incubation period was selected on the basis of time course studies (data not shown). The slides were then dip-washed five times in PBS to remove non-associated PMN cells. Following rinsing, specifically attached cells were quantitated microscopically (with photography) by direct analysis of selected fields. For inhibition experiments, the fibrinogen-coated slides were pre-incubated with various concentrations of Clf40-(40-559), Clf41-(221-559) and α_M I-domain for 2 h at 37°C. Unbound protein was removed by three washes with PBS followed by addition of PMN (5 x 10^5 cells). In variations of the above assay, the inhibitory potential of Clf40-(40-559) on adherence of PMN to glass alone and to glass slides coated with fibronectin (200µg/ml) were also investigated. Coating conditions were performed as for fibrinogen-coated glass slides.

Endothelial cell-PMN adhesion assay.

Human endothelial cells (HUVEC) were obtained from umbilical cord veins using 0.1% collagenase in Krebs-Ringer bicarbonate buffer. The cells were plated

in 60 mm gelatin-coated (0.1% for 2 hours) Petri dishes and grown in RPMI 1640 (Gibco) supplemented with 50 units/ml penicillin, 50 g/ml streptomycin, 10% fetal calf serum and 15 μg/ml endothelial cell growth supplement. Cells were trypsinized briefly and plated in gelatinized-24 wells culture plate as described above. Generally, second-passage cells in monolayer culture were used for experiments, at confluence. After rinsing twice in PBS supplemented by Ca^{2+} and Mg^{2+}, cells were incubated with 1mg/ml human fibrinogen for 1 h at 37°C. Unbound fibrinogen was removed by washing in PBS supplemented by Ca^{2+} and Mg^{2+}. For inhibition experiments, recombinant Clf40-(40-559) (23.7μM) was incubated with the fibrinogen-coated endothelial cell monolayer for 30 min at 37°C. As a control, Clf40-(40-559) was also incubated with the endothelial cells in the absence of fibrinogen. Human PMNs (5 X 10^6 cells) were then added to each well. After an incubation period of 30 min at 37°C, each well was rinsed several times with PBS enriched in Ca^{2+} and Mg^{2+} to remove non-associated PMN, and fixed in 4% paraformaldehyde in PBS. The cells were washed with 0.9% NaCl and stained for 90 sec with the benzidine hydrochloride-based Kaplow stain (12). The staining solution was washed away prior to photography. Adherent neutrophil granules were stained for their contents of MPO with the reagent of Kaplow and appeared dark brown/black under visible light.

αM I-domain-fibrinogen binding assay.

To confirm the ability of recombinant Clf40-(40-559) to interfere with fibrinogen-binding function, a cell-free α_M I-domain-fibrinogen binding assay was developed. Fibrinogen (5μg/ml) in coating solution (0.02% sodium carbonate buffer (pH 9.6)) was absorbed onto 96-well enzyme-linked immunosorbent assay microtiter plates (Sarstedt) for 16 h at 4°C. The plates were washed three times with PBS with 0.05% Tween 20 (PBS-T). A solution of 5% bovine serum albumin (BSA) was added to the wells to block any remaining protein-binding sites. After 2 h at 37°C, the well were washed again three times with PBS-T. Recombinant α_M I-domain, which had previously been biotinylated (with sulfo-NHS-biotin (Pierce)), was added and incubated for 90 min at room temperature. Wells were washed, incubated with alkaline phosphatase-coupled avidin, washed again, developed with substrate, and quantified colorimetrically using a microplate reader. For the inhibition experiments, the immobilized fibrinogen was pre-incubated with increasing concentrations of Clf40-(40-559) for 1 h at room temperature. Unbound protein was removed by three washes with PBS-T.

EXAMPLE 2: ANALYSIS OF THE EXPERIMENTS OF EXAMPLE 1 AND FURTHER TESTING REGARDING THE EFFECT OF CLF40 AND CLF41 ON PMN ADHESION TO FIBRINOGEN

ClfA inhibits PMN leukocyte adhesion to fibrinogen.

Recombinant forms of ClfA encompassing full length region A (Clf40, residues 40-559) or the smallest truncated protein that maintained fibrinogen-binding activity (Clf41, residues 221-559) were tested for their ability to inhibit the adhesion of human PMN to immobilized fibrinogen. The recombinant ClfA

proteins were pre-incubated with immobilized fibrinogen. Unbound protein was removed by washing and the PMN were allowed to interact. As shown in Fig. 2, Clf40-(40-559) inhibited PMN adhesion to fibrinogen in a dose-dependent manner. At the highest concentrations of Clf40-(40-559) tested, PMN adherence was reduced to 20%. of that observed in the absence of inhibitor (n = 4). Interestingly, the minimum fibrinogen-binding truncate of ClfA, Clf41-(221-559), was much less effective at inhibiting PMN adherence to fibrinogen (Fig. 2, inset) even at high concentrations. Several independent studies and approaches have implicated the I-domain of the α_M subunit in the recognition of fibrinogen by $\alpha_M\beta_2$, the major integrin of leukocytes (5, 21, 35). To confirm that activated PMN adherence to fibrinogen is mediated primarily via the integrin, the recombinant I-domain of the α_M subunit was purified and tested for its ability to inhibit PMN fibrinogen-binding activity. As expected, increasing concentrations of recombinant I-domain caused a dose-dependent inhibition of the adhesion of PMN to immobilized fibrinogen (data not shown). At 3.5μM, adhesion to fibrinogen was reduced by 80% (Fig. 2, inset) (n = 4). To demonstrate that Clf40-(40-559) was interfering with fibrinogen-mediated attachment, the ability of the protein to interfere with attachment of activated PMN to fibronectin and glass was analyzed. Consistent with the specificity of inhibition of fibrinogen-mediated adherence, Clf40-(40-559) had no effect on leukocyte adherence to fibronectin-coated slides or to glass alone (Fig. 3). Finally, none of the inhibitors analyzed affected cell viability as assessed by trypan blue exclusion.

ClfA inhibits PMN leukocyte adhesion to fibrinogen.

Fibrinogen bound to ICAM-1 on endothelial cells can facilitate the bridging of these cells to on leukocytes (6, 13). This interaction has been implicated in the inflammatory response by contributing to leukocyte adhesion to the endothelium and subsequent transmigration (14). In addition, Cheung et al. (1991) (4) demonstrated that fibrinogen can act as a bridging molecule in the adherence of *S. aureus* to endothelial cells and implicated the fibrinogen-binding receptor of *S. aureus* as an important factor in this process. We addressed the question of whether ClfA, the major fibrinogen-binding protein of *S. aureus*, could inhibit the attachment of PMN to fibrinogen bound to cultured human umbilical vein endothelium. PMN were allowed to adhere to an endothelial cell monolayer that had been pre-incubated with fibrinogen. The effect of Clf40-(40-559) on this adherence was evaluated microscopically. As shown in Fig. 4A, endothelial cells with bound fibrinogen supported efficient leukocyte adhesion. Inclusion of Clf40-(40-559) protein resulted in a dramatic reduction in leukocyte attachment to (Fig. 4B). In the absence of fibrinogen, there was reduced PMN adhesion to the endothelium probably mediated by ICAM-1 which can interact directly with $\alpha M\beta 2$ and $\alpha L\beta 2$ on leukocytes (Fig. 4C). Clf40-(40-559) had no effect on this fibrinogen-independent endothelial-leukocyte interaction.

ClfA inhibits the interaction between the I-domain of the α_M subunit and fibrinogen.

Many studies have shown that the I-domain of the α-subunit of αMβ2 is an independent structural and functional unit and has many of the binding functions of the intact receptor (15). To further establish the molecular basis of ClfA inhibition of PMN adhesion to fibrinogen, we investigated the inhibitory activity of Clf40-(40-559) on the interaction between recombinant α_M I-domain and immobilized fibrinogen. As shown in Fig. 5, Clf40-(40-559) inhibited the binding of biotinylated α_M I-domain to fibrinogen in a dose-dependent manner. At 5μM concentration, Clf40-(40-559) reduced binding by 60%. In contrast, the control protein (BSA) had no effect on α_M I-domain-fibrinogen interaction. This result indicates that the inhibitory effect of ClfA on PMN leukocyte adherence to fibrinogen is mediated primarily via its effect on the fibrinogen-binding activity of αMα2.

In light of these studies, it has now been shown that the fibrinogen-binding protein of *S. aureus*, ClfA, is an effective and specific inhibitor of PMN interaction with immobilized fibrinogen. Furthermore, the data presented here indicates that ClfA can interfere with PMN fibrinogen-binding activity by interacting with the fibrinogen γ-chain at, or in close proximity, to the binding sites. The three-dimensional structure of the fibrinogen γ-chain module, which became available recently (34), revealed that the two amino acid sequences implicated in interaction (P1, residues 190-202, and P2, residues 377-395) reside adjacent to one another as part of two anti-parallel β-strands. Thus, although the P1 and P2 sites are separated by 178 residues in terms of linear amino acid sequence, the specific folding of the γ-chain module brings these two recognition sequences into

close proximity to form a complex ligand-binding site. The known binding site in fibrinogen for ClfA occurs at the C-terminus of the γ-chain (18). This segment of the structure emerges as an appendage from the globular portion of the γ-chain module at the P1 and P2 sites and is in close proximity to the P2 site. In addition, structural NMR and crystallographic studies provide firm evidence that the C-terminal region of the γ-chain is conformationally flexible which may be functionally important in its biological context (34). It thus appears that when ClfA interacts with the C-terminus of the γ-chain, the protein interferes with access to the P1 and P2 binding sites. This interference may be via steric hindrance blocking accessibility to the recognition sequences.

Alternatively, ClfA may interact directly with one or either of these sites and prevent interaction due to molecular mimicry between the two fibrinogen-binding proteins. Previously, we have shown that ClfA is a potent inhibitor of $\alpha_{IIB}\beta_3$-mediated interaction of platelets with fibrinogen via a molecular mimicry mechanism (18). Our preliminary data indicates that ClfA can recognize a sequence in the fibrinogen γ-chain other than the extreme C-terminus (25). Furthermore, these data suggest that only the recombinant truncate encompassing the full ligand-binding region, Clf40-(40-559), has the capacity to interact with the additional site(s). The minimum fibrinogen-binding truncate, Clf41-(221-559), binds exclusively to the γ-chain C-terminus. Consistent with this proposal is the observation that Clf41-(221-559), while still able to inhibit interaction between PMN and fibrinogen (Fig. 2, inset), had a far reduced ability

when compared to ability of Clf40 to inhibit the interaction between PMN and fibrinogen.

The importance of the interaction between PMN and immobilized fibrinogen in host immune response to infection is well established. Firstly, the interaction is considered crucial in the inflammatory response by contributing to leukocyte adhesion to the endothelium and subsequent transmigration to sites of infection (13, 14, 28). Secondly, in order for PMN to initiate a respiratory burst in response to soluble cytokines, they are required to utilize their β_2 integrins (including $\alpha M\beta 2$) and interact with immobilized matrix molecule such as fibrinogen (22). Furthermore, occupancy of with immobilized fibrinogen profoundly modulates monocyte oxidative responses and phagocytosis of opsonized particles (31). Lastly, fibrinogen and its derivatives are known to directly promote the accumulation of inflammatory cells on biomaterial implants (29, 30). Interestingly, it is well established that biomaterial implants are important sites for the initiation of Staphylococci infection (7). In light of the crucial role PMN play in host response to infection by *S. aureus* and, in particular, the importance of leukocyte interaction with fibrinogen, it is reasonable to suggest that the ability of bacteria to interfere with this interaction would be advantageous and could influence the pathogenic potential of infecting microorganisms. A role for ClfA in pathogenesis has been demonstrated in a rat endocarditis model (20). A *ClfA* mutant of *S. aureus* Newman had a significantly reduced ability to cause infection compared to the wild-type strain. Similarly, a ClfA mutant tested in a mouse septic arthritis model demonstrated a reduced ability to cause arthritic disease (11).

Undoubtedly, reduction in fibrinogen-binding activity plays a major role in the compromised pathogenicity of the *S. aureus ClfA* mutant strains. However, it is possible that reduced ability to interfere with fibrinogen-mediated PMN function in host response to infection may contribute to the decreased virulence of the mutant strains.

Additional levels of complexity in the role *S. aureus* plays in compromising PMN-fibrinogen interaction are possible by considering the presence of additional cell wall-associated and extracellular fibrinogen-binding proteins expressed by the bacteria. However, these proteins do not appear to play a major role in promoting bacterial attachment to immobilized fibrinogen. In addition, two of the better characterized proteins, Efb, an extracellular fibrinogen-binding protein (26) and, ClfB, a cell wall associated MSCRAMM related to ClfA (23), interact with the α-chain and with the α- and β-chains of fibrinogen, receptively. Thus, interference of PMN fibrinogen-binding activity, mediated by the γ-chain, is unlikely. It may be argued that ClfA inhibition of PMN adherence to fibrinogen would be more effective if the protein was extracellular and not bound to the bacterial cell wall.

However, it has been demonstrated that *S. aureus* binds effectively to endothelium via fibrinogen molecules (4), to fibrin blood clots and to fibrinogen-coated biomaterials (7), and the experiments in accordance with the present invention have shown that the ligand-binding regions of ClfA, namely Clf40 (40-559) and Clf41 (221-559), can inhibit the interaction and adhesion between polymorphonuclear leukocytes and immobilized fibrinogen. Accordingly, the present invention can be utilized in those applications, such as the development

of vascular plaque or acute inflammation at the situs of a diseased blood vessel or one that has undergone vascular surgery, wherein reduction of the effects of such adhesion is highly desirable.

EXAMPLE 3: ADDITIONAL INVESTIGATIONS REGARDING PHAGOCYTOSIS OF *S. AUREUS*

Preparation of FITC-labeled bacteria.

The strains of *S. aureus* to be labeled were grown overnight without shaking at 37°C in Mueller-Hinton broth, harvested by centrifugation and washed once with 0.9% NaCl. Following resuspension in 0.1M $NaHCO_3$ (pH 9.0), bacterial cells were incubated with FITC Isomer I (100μg/ml) (Molecular Probes, Leiden, NL) in 0.5M $NaHCO_3$ buffer (pH 9.0), for 1 hour at 37°C. Following conjugation, the cells were washed and fixed with 0.5% (v/v) formaldehyde in PBS for 1 hour at room temperature. After one wash with PBS, the cells were resuspended in PBS with 1% BSA. Before use, the FITC-conjugated bacteria were stored at 4°C.

Phagocytosis of FITC-conjugated *S. aureus* by human PMN.

FITC-conjugated *S. aureus* strains (Newman wild-type, Newman Δ*clfA*, Newman Δ*spa* and Newman Δ*clfA* Δ*spa*) (1 x 10^7 cells/ml) were suspended in PBS with or without 10% normal human serum (NHS) from healthy donors. Following incubation for 30 min. at 37°C, the cells were washed twice in PBS with 1% BSA and resuspended in the same buffer. To ensure the cells were in

suspension as single cells, the *S. aureus* cell suspension was sonicated gently and evaluated microscopically. A 500µl volume of FITC-labeled *S. aureus* suspension (1 x 10^7 cells/ml) was mixed with 500µl of purified human PMN isolated from whole blood from healthy donors (2 x 10^6 cells/ml) (1A). Following incubation for 0 or 5 min at 37°C, phagocytosis was terminated by placing the PMN-*S. aureus* cell mixture on ice. After 5 min, the cell suspension was centrifuged briefly and the pellet was resuspended in 500µl of PBS with 2.5% BSA for analysis by flow cytometry. To distinguish between bacteria adherent to PMN and bacteria internalized within PMN, trypan blue was added to quench fluorescence due to extracellular FITC-conjugated bacteria. As trypan blue cannot enter PMN cells, fluorescence due to phagocytosed bacteria was not affected by addition of the dye.

Flow cytometric analysis was performed on a Becton Dickinson FACScan cytometer with 488nm excitation light and emission collected through a 520/20nm bandpass filter. In general, 5000 cells were analyzed in the FI-1 channel, and light scatter and fluorescence signals were collected with fixed amplification. The threshold settings for non-specific fluorescence were obtained by using PMN cells in the absence of FITC-labeled bacteria. Histograms of fluorescent FSC/SSC-gated cells were obtained. The level of phagocytosis (expressed as arbitrary fluorescence units (AFU)) was determined according to the following equation:

1AFU = (mean FI-1 height of positive events (cells)) x (% of positive events (cells))/1000

Assessment of results.

S. aureus is a gram-positive bacterium, which is not sensitive to the lytic effect of complement but is attacked through the surface deposition of C3b. After initial deposition of the C3b molecule, the amplification system of the alternative pathway rapidly increases the deposition of C3b, making the bacteria sensitive to phagocytosis by phagocytic cells. The main C3b receptor on these cells is the integrin, (CR3). Additionally, coating of bacteria with serum immunoglobulins allows phagocytic cells to engulf the microorganism via the antibody Fc receptors on their surface.

We investigated the ability of two surface adhesins of *S. aureus*, the fibrinogen-binding protein, ClfA, and the IgG-binding protein, protein A, in protecting the bacteria from opsonophagocytosis by human PMNs. An earlier study implicated protein A in rendering *S. aureus* less susceptible to antibody-mediated phagocytosis (37). In this study using isogenic site-specific mutants of *S. aureus,* it was demonstrated that ClfA-and protein A-deficient strains were taken up more efficiently by PMNs compared to the wild-type strain (Fig. 6). A double mutant *S. aureus* strain in which both protein A and ClfA were deleted was dramatically more susceptible (by 200%) to phagocytosis by PMNs compared to the wild-type strain.

These data indicate that both protein A and ClfA contribute to protecting *S. aureus* from phagocytosis by human PMNs, and thus the use of these binding regions in accordance with the methods of the present invention may provide for enhanced bacterial phagocytosis. The finding that protein A inhibits PMN

phagocytosis was not unexpected as protein A is known to interact with the Fc domain of IgG (38). As this region of the IgG molecule is also the binding site for the Fc receptors on PMNs, bound IgG on the surface of the bacteria will be unable to promote antibody-mediated phagocytosis. However, the discovery by the present inventors that ClfA interferes with phagocytosis is novel and has not been previously reported. The interaction of ClfA and fibrinogen could serve to coat the surface of the bacteria with fibrinogen and thus mask binding sites for opsonins present in normal sera, and ClfA may interact with iC3b by a mechanism similar to its interaction with αMβ2.

In this study, it has been demonstrated that ClfA inhibits αMβ2 fibrinogen-binding activity by interacting with, or in close proximity to, the αMβ2-binding site in fibrinogen. The notion that ClfA shares functional similarities to was further strengthened when the ligand-binding region of ClfA was shown to have sequence homology to an integrin-like protein (αInt1p) from *Candida albicans,* a protein which also shares functional and sequence homologies with αMβ2 (39). By interacting with iC3b at the αMβ2 binding site, ClfA could utilize molecular mimicry to inhibit complement-mediated phagocytosis.

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WHAT IS CLAIMED IS:

1. A method of inhibiting the adhesion of leukocytes to fibrinogen comprising administering an effective amount of a protein selected from the group consisting of Clf40 and Clf41 so that the protein inhibits said adhesion at a situs where inhibition of such adhesion is desired.

2. A method according to Claim 1 wherein the administration of the protein is directly at the situs wherein inhibition of leukocyte adhesion is desired.

3. A method according to Claim 1 wherein the administration of the protein is systemic and the protein will be systemically transmitted to the situs wherein inhibition of leukocyte adhesion is desired.

4. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are natural or recombinant proteins from staphylococcal bacteria.

5. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are natural or recombinant proteins from *Staphylococcus aureus*.

6. A method according to Claim 1 wherein the Clf40 and Clf41 proteins are in the form of active fragments or portions of the natural or recombinant proteins.

7. A method according to Claim 1 wherein the protein is administered at the situs of a vascular injury.

8. A method according to Claim 1 wherein the protein is administered following a vascular surgical operation.

9. A method according to Claim 1 wherein the protein is administered at the situs of a vascular implant.

10. A composition for inhibiting the adhesion of leukocytes to fibrinogen comprising an isolated protein selected from the group consisting of Clf40 and Clf41 and a pharmaceutically acceptable vehicle, excipient or carrier.

11. An antibody raised against an isolated and purified form of a protein selected from the group consisting of Clf40 and Clf41.

12. A pharmaceutical composition comprising an antibody according to Claim 9 and a pharmaceutically acceptable vehicle, excipient or carrier.

13. A vaccine comprising an immunologically effective amount of a protein selected from the group consisting of Clf40 and Clf41 and a pharmaceutically acceptable carrier or excipient.

14. A vaccine according to Claim 13 wherein the proteins are from staphylococcal bacteria.

15. A vaccine according to Claim 13 wherein the proteins are from *Staphylococcus aureus*.

16. A vaccine according to Claim 13 wherein the proteins are in the form of immunogenic fragments or portions of the proteins.

17. A vaccine according to Claim 13 further comprising a pharmaceutically acceptable adjuvant.

18. A method of generating an immune response in a host comprising administering to said host an immunologically effective amount of the composition of Claim 10.

19. A method of enhancing the immunogenic response in a patient comprising administering to said patient an immunogenically effective amount of a pharmaceutical composition selected from the group consisting of a composition

comprising an comprising a Clf40 or Clf41 protein and a pharmaceutically acceptable vehicle, excipient or carrier, and a composition comprising an antibody according to Claim 9 and a pharmaceutically acceptable vehicle, excipient or carrier.

20. A method of increasing phagocytosis in a patient comprising administering to said patient a pharmaceutical composition selected from the group consisting of a composition comprising an comprising a Clf40 or Clf41 protein and a pharmaceutically acceptable vehicle, excipient or carrier, and a composition comprising an antibody according to Claim 11 and a pharmaceutically acceptable vehicle, excipient or carrier, in an amount effective to increase phagocytosis in a patient.

21. A vaccine comprising a pharmaceutically acceptable formulation comprising nucleic acid encoding Clf40 or Clf41 and a pharmaceutically acceptable vehicle, carrier or excipient.

22. A kit for detecting Clf40 or Clf41 or antibodies thereto, comprising an isolated Clf40 or Clf41 protein and means to identify the binding thereto of an antibody in a sample suspected of containing such antibodies, or an antibody to an isolated Clf40 or Clf41 protein and means to identify the binding thereto of a Clf40 or Clf41 protein in a sample suspected of containing such proteins.

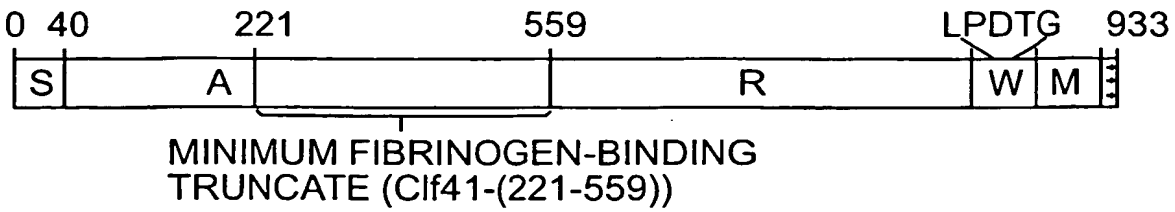

0 40
221
559
LPDTG
933
S
A
R
W
M
MINIMUM FIBRINOGEN-BINDING
TRUNCATE (Clf41-(221-559))

FIG. 1

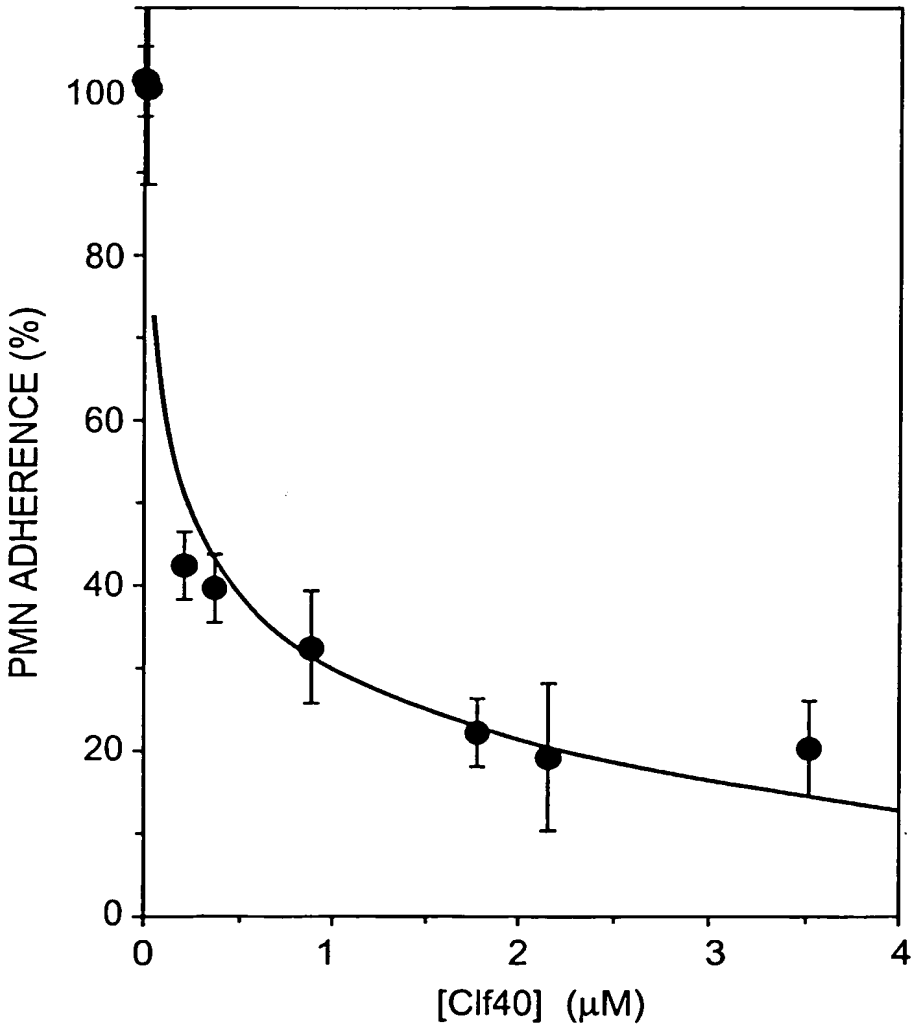
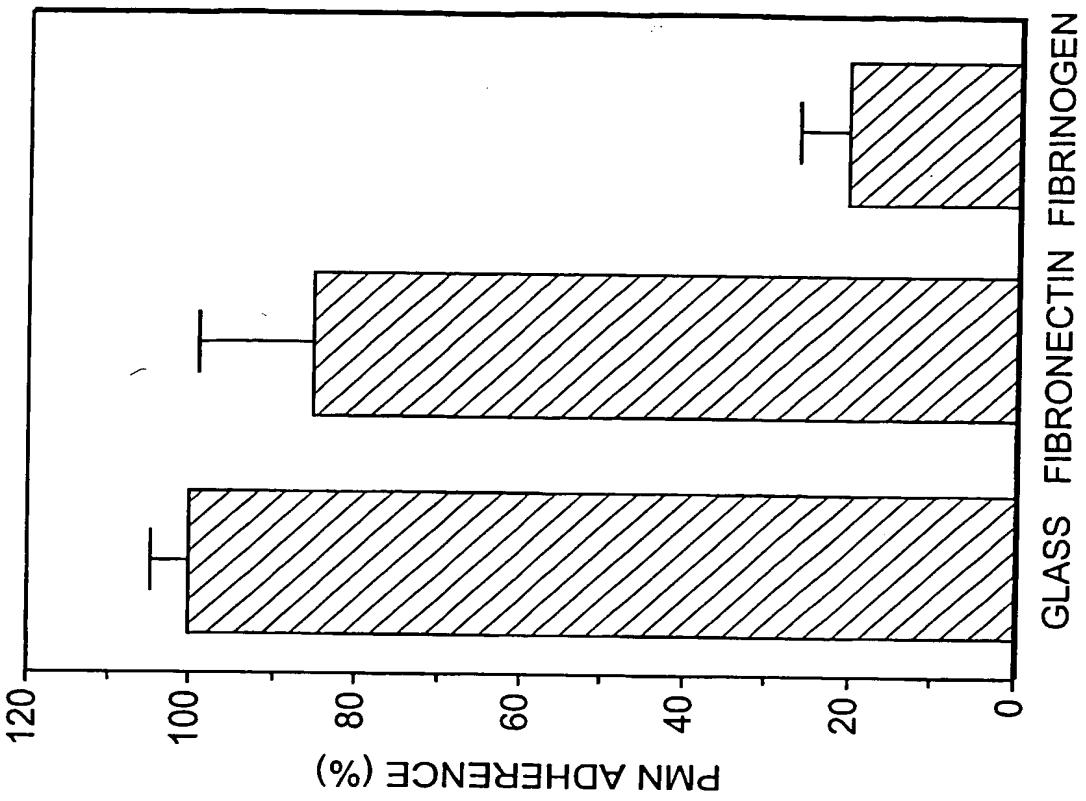

PMN ADHERENCE (%)
100
80
60
40
20
0
0
1
2
3
4
[Clf40] (μM)

FIG. 2


PMN ADHERENCE (%)
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20
40
60
80
100
120
GLASS
FIBRONECTIN
FIBRINOGEN

FIG. 3

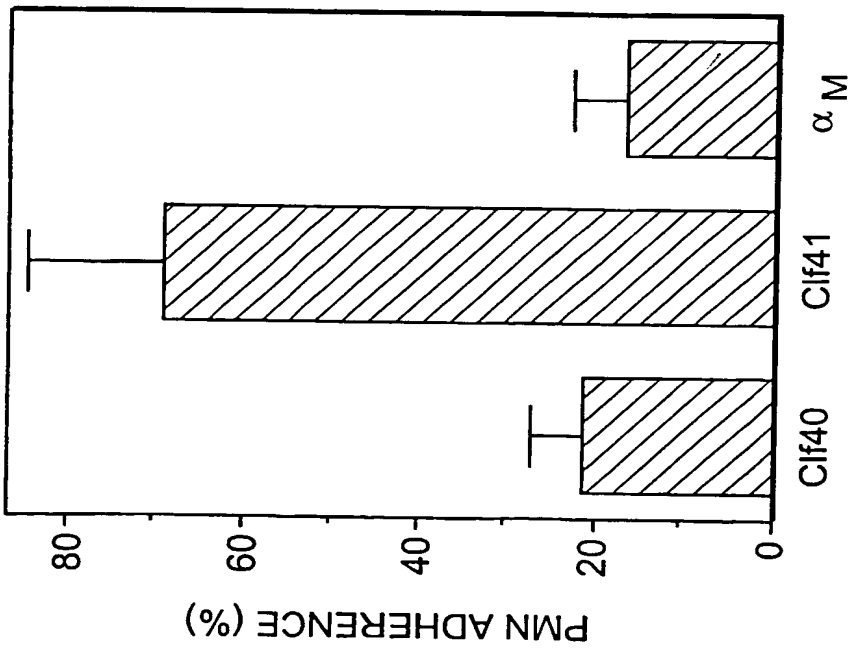
PMN ADHERENCE (%)
0
20
40
60
80
Clf40
Clf41
α_M

FIG. 2A

FIG. 4C

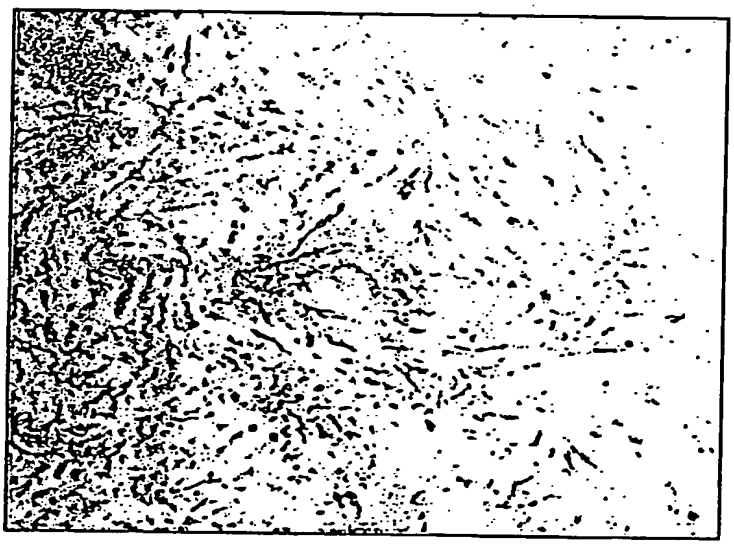

FIG. 4B

FIG. 4A

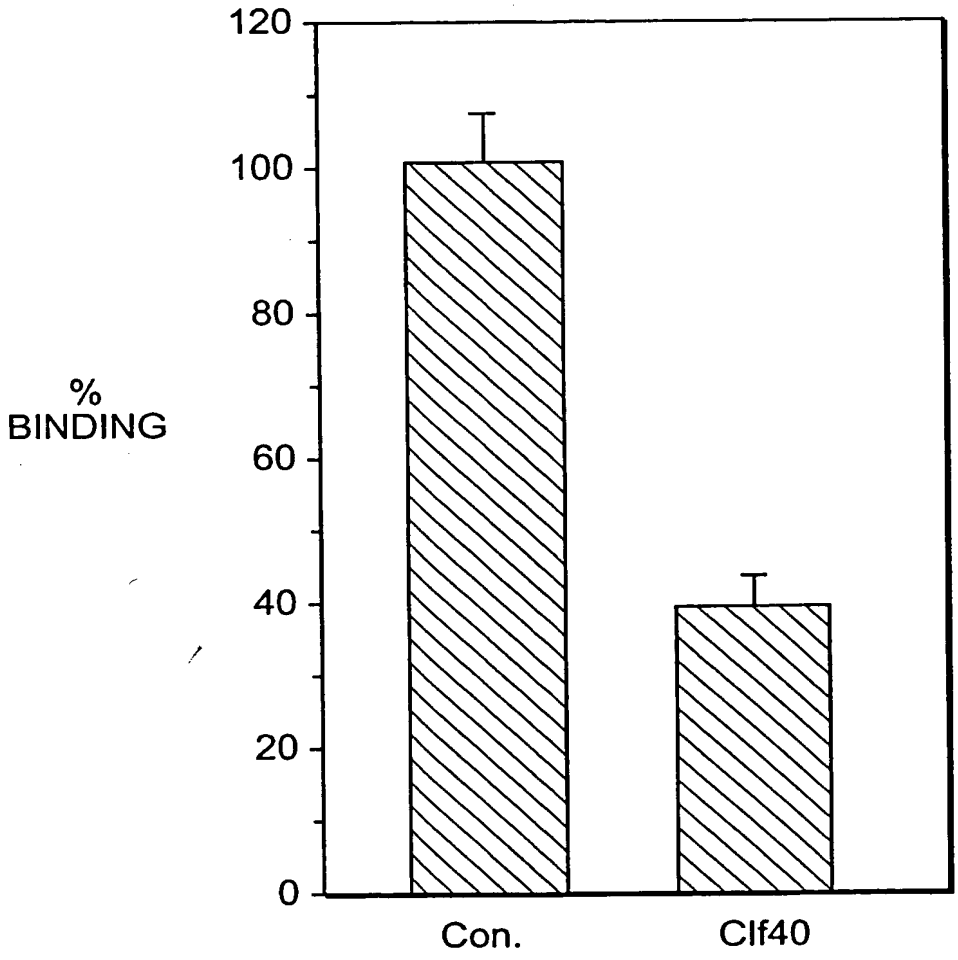

FIG. 5

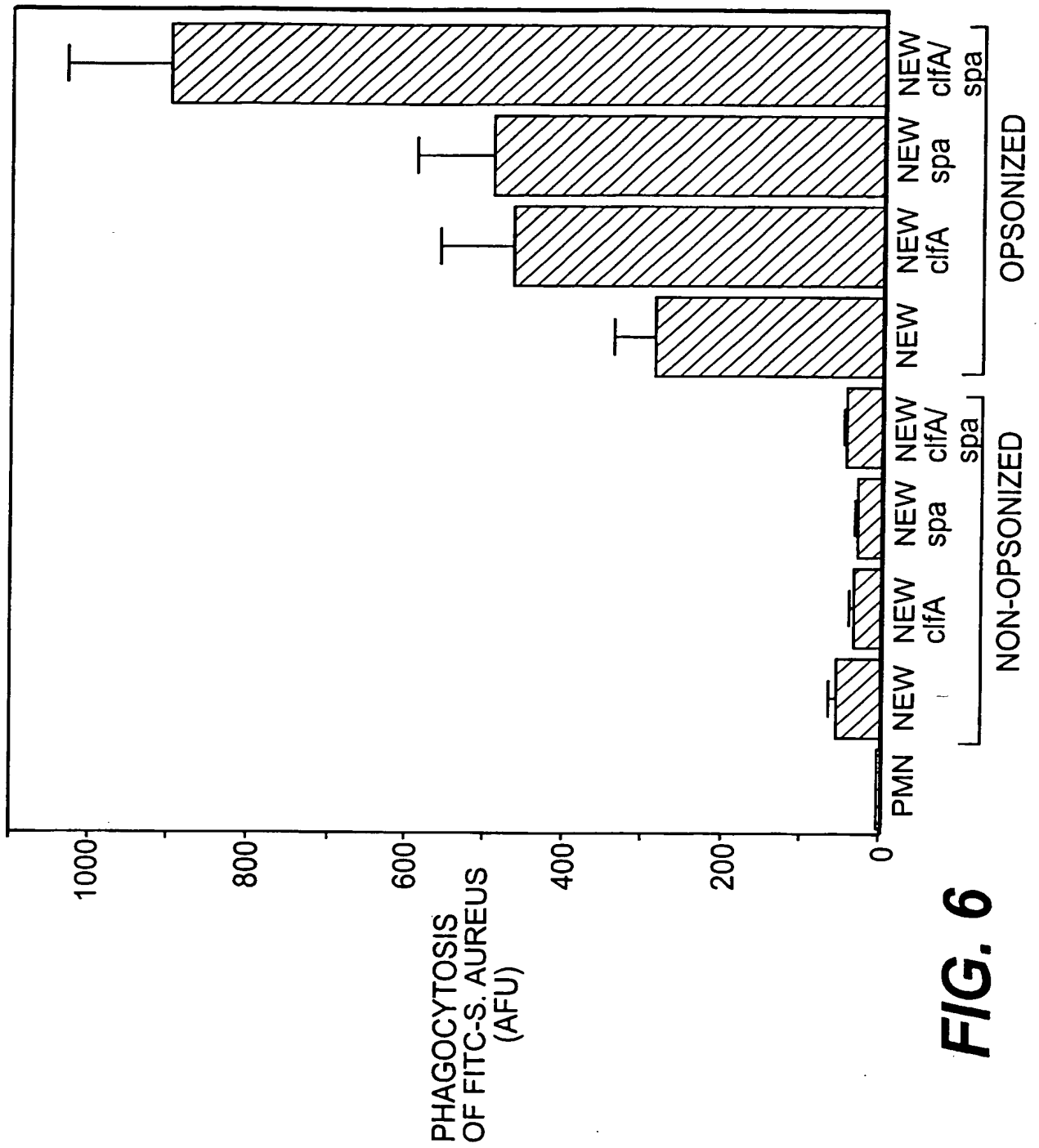
PHAGOCYTOSIS OF FITC-S. AUREUS (AFU)
0
200
400
600
800
1000
PMN
NEW
NEW clfA
NEW spa
NEW clfA/ spa
NEW
NEW clfA
NEW spa
NEW clfA/ spa
NON-OPSONIZED
OPSONIZED

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/11685

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C07K 1/00, 14/00, 17/00
US CL : 530/350; 536/23.7; 435/69.1, 69.3 252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.7; 435/69.1, 69.3 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

none

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST: leukocyte adhesion, phagocytosis, fibrinogen, immunogenic response, clf40 and clf41 proteins, staphylococcus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 6,008,341 A (FOSTER et al.) 28 December 1999, see entire document.	1-22

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 JULY 2000	11 AUG 2000
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